

ATTENUATED HUMAN-BOVINE CHIMERIC PARAINFLUENZA VIRUS (PIV) VACCINES

CROSS-REFERENCES TO RELATED APPLICATIONS

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This application claims the benefit of United States Provisional Patent Application Serial No. 60/143,134, filed by Bailly et al. on July 9, 1999.

2/10/99

BACKGROUND OF THE INVENTION

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Human parainfluenza virus type 3 (HPIV3) is a common cause of serious lower respiratory tract infection in infants and children less than one year of age. It is second only to respiratory syncytial virus (RSV) as a leading cause of hospitalization for viral lower respiratory tract disease in this age group (Collins et al., in B. N. Fields Virology, p. 1205-1243, 3rd ed., vol. 1., Knipe et al., eds., Lippincott-Raven Publishers, Philadelphia, 1996; Crowe et al., Vaccine 13:415-421, 1995; Marx et al., J. Infect. Dis. 176:1423-1427, 1997, all incorporated herein by reference). Infections by this virus result in substantial morbidity in children less than 3 years of age. HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup) and also can cause severe pneumonia and bronchiolitis (Collins et al., 1996, *supra*). In a long term study over a 20-year period, HPIV1, HPIV2, and HPIV3 were identified as etiologic agents for 6.0, 3.2, and 11.5%, respectively, of hospitalizations for respiratory tract disease accounting in total for 18% of the hospitalizations, and, for this reason, there is a need for an effective vaccine (Murphy et al., Virus Res. 11:1-15, 1988). The parainfluenza viruses have also been identified in a significant proportion of cases of virally-induced middle ear effusions in children with otitis media (Heikkinen et al., N. Engl. J. Med. 340:260-264, 1999, incorporated herein by reference). Thus, there is a need to produce a vaccine against these viruses that can prevent the serious lower respiratory tract disease and the otitis media that accompanies these HPIV infections. HPIV1, HPIV2, and HPIV3 are distinct serotypes that do not elicit significant cross-protective immunity. The major protective antigens of PIVs are the hemeagglutinin (HN) and fusion (F) glycoproteins, which mediate viral attachment, penetration and release. Protection against reinfection is mediated primarily by virus-neutralizing antibodies.

Despite considerable efforts to develop effective vaccine therapies against HPIV, approved vaccine agents that prevent HPIV related illness have not yet been achieved. The most promising prospects to date are live attenuated vaccine viruses since these have been shown to be efficacious in non-human primates even in the presence of passively transferred antibodies, an experimental situation that simulates that present in the very young infant who possesses maternally acquired antibodies (Crowe et al., 1995, *supra*; and Durbin et al., J. Infect. Dis. 179:1345-1351, 1999a; each incorporated herein by reference). Two live attenuated PIV3 vaccine candidates, a temperature-sensitive (*ts*) derivative of the wild type PIV3 JS strain (designated PIV3*cp45*) and a bovine PIV3 (BPIV3) strain, are undergoing clinical evaluation (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., 1995a, *supra*; Karron et al., 1995b, *supra*; each incorporated herein by reference). The BPIV3 vaccine candidate is attenuated, genetically stable and immunogenic in human infants and children. A second PIV3 vaccine candidate, JS *cp45*, is a cold-adapted mutant of the JS wildtype (wt) strain of HPIV3 (Karron et al., 1995b, *supra*; and Belshe et al., J. Med. Virol. 10:235-242, 1982a; each incorporated herein by reference). This live, attenuated, cold-passaged (*cp*) PIV3 vaccine candidate exhibits temperature-sensitive (*ts*), cold-adaptation (*ca*), and attenuation (*att*) phenotypes, which are stable after viral replication *in vivo*. The *cp45* virus is protective against human PIV3 challenge in experimental animals and is attenuated, genetically stable, and immunogenic in seronegative human infants and children (Belshe et al., 1982a, *supra*; Belshe et al., Infect. Immun. 37:160-165, 1982b; Clements et al., J. Clin. Microbiol. 29:1175-1182, 1991; Crookshanks et al., J. Med. Virol. 13:243-249, 1984; Hall et al., Virus Res. 22:173-184, 1992; Karron et al., 1995b, *supra*; each incorporated herein by reference). Because these PIV3 candidate vaccine viruses are biologically derived there is no proven method for adjusting their level of attenuation as will likely be necessary for broad clinical application.

To facilitate development of PIV vaccine candidates, recombinant DNA technology has recently made it possible to recover infectious negative-strand RNA viruses from cDNA (for reviews, see Conzelmann, J. Gen. Virol. 77:381-389, 1996; Palese et al., Proc. Natl. Acad. Sci. U.S.A. 93:11354-11358, 1996; each incorporated herein by reference). In this context, rescue of recombinant viruses has been reported for infectious respiratory syncytial virus (RSV), rabies virus (RaV), simian virus 5 (SV5), rinderpest virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles virus (MeV), mumps virus (MuV) and Sendai virus (SeV) from cDNA-encoded

antigenomic RNA in the presence of essential viral proteins (see, e.g., Garcin et al., EMBO J. 14:6087-6094, 1995; Lawson et al., Proc. Natl. Acad. Sci. U.S.A. 92:4477-4481, 1995; Radecke et al., EMBO J. 14:5773-5784, 1995; Schnell et al., EMBO J. 13:4195-4203, 1994; Whelan et al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-8392, 1995; Hoffman et al., J. Virol. 71:4272-4277, 1997; Kato et al., Genes to Cells 1:569-579, 1996; Roberts et al., Virology 247:1-6, 1998; Baron et al., J. Virol. 71:1265-1271, 1997; International Publication No. WO 97/06270; Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567, 1995; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to published International Application No. WO 98/02530 and priority U.S. Provisional Application Nos. 60/047,634, filed May 23, 1997, 60/046,141, filed May 9, 1997, and 60/021,773, filed July 15, 1996); U.S. Patent Application Serial No. 09/291,894, filed on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999; U.S. Provisional Patent Application Serial No. 60/143,097, filed by Bucholz et al. on July 9, 1999; Juhasz et al., J. Virol. 71:5814-5819, 1997; He et al. Virology 237:249-260, 1997; Peters et al. J. Virol. 73:5001-5009, 1999; Whitehead et al., Virology 247:232-239, 1998a; Whitehead et al., J. Virol. 72:4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; Bucholz et al. J. Virol. 73:251-259, 1999; Whitehead et al., J. Virol. 73:3438-3442, 1999, and Clarke et al., J. Virol. 74:4831-4838, 2000; each incorporated herein by reference in its entirety for all purposes).

In more specific regard to the instant invention, a method for producing HPIV with a wt phenotype from cDNA was recently developed for recovery of infectious, recombinant HPIV3 JS strain (see, e.g., Durbin et al., Virology 235:323-332, 1997a; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application Serial No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application Serial No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, these disclosures allow for genetic manipulation of viral cDNA clones to determine the genetic basis of phenotypic changes in biological mutants, e.g., which mutations in the HPIV3 cp45 virus specify its ts, ca and att phenotypes, and which gene(s) or genome segment(s) of BPIV3 specify its attenuation phenotype. Additionally, these and related disclosures render it feasible to construct novel PIV vaccine candidates having a wide range of different mutations and to evaluate their level of attenuation, immunogenicity and phenotypic stability (see also, U.S. Provisional Patent Application Serial No. 60/143,134,

filed by Bailly et al. on July 9, 1999; and U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999; each incorporated herein by reference).

Thus, infectious wild type recombinant PIV3 (r)PIV3, as well as a number of *ts* derivatives, have now been recovered from cDNA, and reverse genetics systems have been used to generate infectious virus bearing defined attenuating mutations and to study the genetic basis of attenuation of existing vaccine viruses. For example, the three amino acid substitutions found in the L gene of *cp45*, singularly or in combination, have been found to specify the *ts* and attenuation phenotypes. Additional *ts* and attenuating mutations are present in other regions of the PIV3 *cp45*. In addition a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45*L (Skiadopoulos et al., J. Virol. 72:1762-1768, 1998; Tao et al., J. Virol. 72:2955-2961, 1998; Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.*cp45*L was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1.*cp45*, has been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations that occur in HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al., Vaccine 18:503-510, 1999a).

BPIV3, which is antigenically-related to HPIV3, offers an alternative approach to the development of a live attenuated virus vaccine for HPIV1, HPIV2, and HPIV3. The first vaccine used in humans, live vaccinia virus believed to be of bovine origin, was developed by Jenner almost 200 years ago for the control of smallpox. During the ensuing two centuries, vaccinia virus was successful in controlling this disease and played an essential role in the final eradication of smallpox. In this "Jennerian" approach to vaccine development, an antigenically-related animal virus is used as a vaccine for humans. Animal viruses that are well adapted to their natural host often do not replicate efficiently in humans and hence are attenuated. At present, there is a lack of a thorough understanding regarding the genetic basis for this form of host range restriction. Evolution of a virus in its mammalian or avian host results in significant divergence of nucleotide (nt) and amino acid sequences from that of the corresponding sequences in the related human virus. This divergent sequence, consisting of a large number of sequence differences, specifies the host range attenuation phenotype. Having

an attenuation phenotype which is based on numerous sequence differences is a desirable property in a vaccine virus since it should contribute to the stability of the attenuation phenotype of the animal virus following its replication in humans.

The recently licensed quadrivalent rotavirus is an example of the Jennerian approach to vaccine development in which a nonhuman rotavirus strain, the rhesus rotavirus (RRV), was found to be attenuated in humans and protective against human serotype 3 to which it is antigenically highly related (Kapikian et al., Adv. Exp. Med. Biol. 327:59-69, 1992). Since there was a need for a multivalent vaccine that would induce resistance to each of the four major human rotavirus serotypes, the Jennerian approach was modified by constructing three reassortant viruses using conventional genetic techniques of gene reassortment in tissue culture. Each single gene reassortant virus contained 10 RRV genes plus a single human rotavirus gene that coded for the major neutralization antigen (VP7) of serotype 1, 2, or 4. The intent was to prepare single gene substitution RRV reassortants with the attenuation characteristics of this simian virus and the neutralization specificity of human rotavirus serotype 1, 2, or 4. The quadrivalent vaccine based on the host range restriction of the simian RRV in humans provided a high level of efficacy against human rotavirus infection in infants and young children (Perez-Schael et al., N. Engl. J. Med. 337:1181-1187, 1997). However, the vaccine virus retains mild reactogenicity in older seronegative infants lacking maternal antibody, therefore a second generation Jennerian vaccine, based on the UK strain of bovine rotavirus, is being developed to replace the RRV vaccine (Clements-Mann et al., Vaccine 17:2715-2725, 1999).

The Jennerian approach also is being explored to develop vaccines for parainfluenza type 1 virus and for hepatitis A virus which are attenuated and immunogenic in non-human primates (Emerson et al., J. Infect. Dis. 173:592-597, 1996; Hurwitz et al., Vaccine 15:533-540, 1997). The Jennerian approach was used for the development of a live attenuated vaccine for influenza A virus but it failed to produce a consistently attenuated vaccine for use in humans (Steinhoff et al., J. Infect. Dis. 163:1023-1028, 1991). As another example, reassortant viruses that contain two gene segments encoding the hemagglutinin and neuraminidase surface glycoproteins from a human influenza A virus and the six remaining gene segments from an avian influenza A virus were attenuated in humans (Clements et al., J. Clin. Microbiol. 27:219-222, 1989; Murphy et al., J. Infect. Dis. 152:225-229, 1985; and Snyder et al., J. Clin. Microbiol. 23:852-857, 1986). This indicated that one or more of the six gene segments of the avian

virus attenuated the avian-human influenza A viruses for humans. The genetic determinants of this attenuation were mapped using reassortant viruses possessing a single gene segment from an attenuating avian influenza A virus and the remaining genes from a human strain. It was shown that the nonstructural (NS), polymerase (PB1, PB2) and M genes contributed to the attenuation phenotype of avian influenza A viruses in humans (Clements et al., J. Clin. Microbiol. 30:655-662, 1992).

In another study, the severe host range restriction of bovine respiratory syncytial virus (BRSV) for replication in chimpanzees was only slightly alleviated by replacement of the BRSV F and G glycoproteins with their HRSV counterparts. This indicated that F and G are involved in this host range restriction, but that one or more additional bovine RSV genes are also involved (Buchholz et al., J. Virol. 74:1187-1199, 2000). This illustrates that more than one gene can contribute in unpredictable ways to the host range restriction phenotype of a mammalian or avian virus in primates.

The instant invention provides a new basis for attenuating a wild type or mutant parental virus for use as a vaccine against HPIV, in which attenuation is based completely or in part on host range effects, while at least one or more of the major neutralization and protective antigenic determinant(s) of the chimeric virus is homologous to the virus against which the vaccine is directed. The HN and F proteins of BPIV3 are each approximately 80% related by amino acid sequence to their corresponding HPIV3 proteins (Suzu et al., Nucleic Acids Res. 15:2945-2958, 1987, incorporated herein by reference) and 25% related by antigenic analysis (Coelingh et al., J. Virol. 64:3833-3843, 1990; Coelingh et al., J. Virol. 60:90-96, 1986; van Wyke Coelingh et al., J. Infect. Dis. 157:655-662, 1988, each incorporated herein by reference). Previous studies indicated that two strains of BPIV3, the Kansas (Ka) strain and the Shipping Fever (SF) prototype strain, were attenuated for the upper and lower respiratory tract of rhesus monkeys, and one of these, the Ka strain, was attenuated in chimpanzees (van Wyke Coelingh et al., 1988, *supra*, incorporated herein by reference). Immunization of nonhuman primates with the Ka virus induced antibodies reactive with HPIV3 and induced resistance to the replication of the human virus in the upper and the lower respiratory tract of monkeys (*id.*) Subsequent evaluation of the Ka strain in humans indicated that the virus was satisfactorily attenuated for seronegative infants, and it retained the attenuation phenotype following replication in fully susceptible infants and children (Karron et al., 1996, *supra*; and Karron et al., 1995a, *supra*; each incorporated herein by reference). Its major advantages therefore were that it was satisfactorily attenuated for fully susceptible

seronegative infants and children, and its attenuation phenotype was stable following replication in humans.

However, the level of serum hemagglutination-inhibiting antibodies reactive with HPIV3 induced in seronegative vaccinees who received $10^{5.0}$ tissue culture infectious dose₅₀ (TCID)₅₀ of the Ka strain of BPIV3 was 1:10.5, which was three-fold lower than similar vaccinees who received a live attenuated HPIV3 vaccine (Karron et al., 1995a, *supra*; and Karron et al., 1995b, *supra*; each incorporated herein by reference). This lower level of antibodies to the human virus induced by BPIV3 reflected in large part the antigenic divergence between HPIV3 and BPIV3 (Karron et al., 1996, *supra*; and Karron et al., 1995a, *supra*; each incorporated herein by reference). Studies to determine the efficacy of the Ka vaccine candidate against HPIV3 in humans have not been performed, but it is likely that this reduced level of antibodies reactive with HPIV3 will be reflected in a reduced level of protective efficacy.

Although it is clear that BPIV3 has host range genes that restrict replication in the respiratory tract of rhesus monkeys, chimpanzees and humans, it remains unknown which of the bovine proteins or noncoding sequences contribute to this host range restriction of replication. It is possible that any of the BPIV3 proteins or noncoding sequences may confer a host range phenotype. It is not possible to determine in advance which genes or genome segments will confer an attenuation phenotype. This can only be accomplished by systematic substitution of BPIV3 coding and non-coding sequences for their HPIV3 counterparts and by evaluation of the recovered HPIV3/BPIV3 chimeric viruses in seronegative rhesus monkeys or humans.

Despite the numerous advances toward development of effective vaccine agents against PIV serotypes 1, 2, and 3, there remains a clear need in the art for additional tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to PIV, particularly illnesses among infants and children due to infection by HPIV. Among the remaining challenges in this context is the need for additional tools to generate suitably attenuated, immunogenic and genetically stable vaccine candidates for use in diverse clinical settings. To facilitate these goals, existing methods for identifying and incorporating attenuating mutations into recombinant vaccine strains must be expanded. Furthermore, it is recognized that methods and compositions for designing vaccines against human PIV can be implemented as well to design novel vaccine candidates for veterinary use. Surprisingly,

the present invention fulfills these needs and provides additional advantages as described hereinbelow.

SUMMARY OF THE INVENTION

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The present invention provides human-bovine chimeric parainfluenza viruses (PIVs) that are infectious and attenuated in humans and other mammals. In related aspects, the invention provides novel methods for designing and producing attenuated, human-bovine chimeric PIVs that are useful in various compositions to generate a desired immune response against PIV in a host susceptible to PIV infection. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a chimeric PIV genome or antigenome including a partial or complete human or bovine PIV “background” genome or antigenome combined or integrated with one or more heterologous gene(s) or genome segment(s) of a different PIV virus. Also provided within the invention are methods and compositions incorporating human-bovine chimeric PIV for prophylaxis and treatment of PIV infection.

The invention thus involves a method for developing live attenuated PIV vaccine candidates based on chimeras between HPIVs and BPIV3. Chimeras are generated using a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and are propagated in the same manner as if they were biologically-derived viruses. Chimeric human-bovine PIV of the invention are recombinantly engineered to incorporate nucleotide sequences from both human and bovine PIV strains to produce an infectious, chimeric virus or subviral particle. In this manner, candidate vaccine viruses are recombinantly engineered to elicit an immune response against PIV in a mammalian host susceptible to PIV infection, including humans and non-human primates. Human-bovine chimeric PIV according to the invention may be engineered to elicit an immune response to a specific PIV, e.g., HPIV3, or a polyspecific response against multiple PIVs, e.g., HPIV1 and HPIV3. Additional chimeric viruses can be designed in accordance with the teachings herein which serve as vectors for antigens of non-PIV pathogens, for example respiratory syncytial virus (RSV) or measles virus.

Exemplary human-bovine chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome comprising both human and bovine polynucleotide

sequences, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

Chimeric human-bovine PIV of the invention include a partial or complete “background” PIV genome or antigenome derived from or patterned after a human or bovine PIV strain or subgroup virus combined with one or more heterologous gene(s) or genome segment(s) of a different PIV strain or subgroup virus to form the human-bovine chimeric PIV genome or antigenome. In preferred aspects of the invention, chimeric PIV incorporate a partial or complete human PIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a bovine PIV.

The partial or complete background genome or antigenome typically acts as a recipient backbone or vector into which are imported heterologous genes or genome segments of the counterpart, human or bovine PIV. Heterologous genes or genome segments from the counterpart, human or bovine PIV represent “donor” genes or polynucleotides that are combined with, or substituted within, the background genome or antigenome to yield a human-bovine chimeric PIV that exhibits novel phenotypic characteristics compared to one or both of the contributing PIVs. For example, addition or substitution of heterologous genes or genome segments within a selected recipient PIV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity, or other desired phenotypic changes as compared with a corresponding phenotype(s) of the unmodified recipient and/or donor.

Genes and genome segments that may be selected for use as heterologous substitutions or additions within human-bovine chimeric PIV of the invention include genes or genome segments encoding a PIV N, P, C, D, V, M, F, SH (where appropriate), HN and/or L protein(s) or portion(s) thereof. In addition, genes and genome segments encoding non-PIV proteins, for example, an SH protein as found in mumps and SV5 viruses, may be incorporated within human-bovine PIV of the invention. Regulatory regions, such as the extragenic 3' leader or 5' trailer regions, and gene-start, gene-end, intergenic regions, or 3' or 5' non-coding regions, are also useful as heterologous substitutions or additions.

Preferred human-bovine chimeric PIV vaccine candidates of the invention

bear one or more of the major antigenic determinants of HPIV3 in a background which is attenuated by the substitution or addition of one or more BPIV3 genes or genome segments. The major protective antigens of PIVs are their HN and F glycoproteins, although other proteins can also contribute to a protective immune response. In certain
5 embodiments, the background genome or antigenome is an HPIV genome or antigenome, e.g., an HPIV3, HPIV2, or HPIV1 background genome or antigenome, to which is added or into which is substituted one or more BPIV gene(s) or genome segment(s), preferably from BPIV3. In one exemplary embodiment described below, an ORF of the N gene of a
10 antigenome may be a BPIV genome or antigenome which is combined with one or more genes or genome segments encoding a HPIV3, HPIV2, or HPIV1 glycoprotein, glycoprotein domain or other antigenic determinant.

In accordance with the methods of the invention, any BPIV gene or genome segment, singly or in combination with one or more other BPIV genes, can be
15 combined with HPIV sequences to give rise to a human-bovine chimeric PIV vaccine candidate. Any HPIV, including different strains of a particular HPIV serotype, e.g., HPIV3 will be a reasonable acceptor for attenuating BPIV gene(s). In general, the HPIV3 gene(s) or genome segment(s) selected for inclusion in a human-bovine chimeric PIV for use as a vaccine against human PIV will include one or more of the HPIV protective
20 antigens such as the HN or F glycoproteins.

In exemplary aspects of the invention, human-bovine chimeric PIV bearing one or more bovine gene(s) or genome segment(s) exhibits a high degree of host range restriction, e.g., in the respiratory tract of mammalian models of human PIV infection such as non-human primates. In exemplary embodiments a human PIV is
25 attenuated by the addition or substitution of one or more bovine gene(s) or genome segment(s) to a partial or complete human, e.g., HPIV3, PIV background genome or antigenome. In one example, the HPIV3 N gene is substituted by the BPIV3 N gene to yield a novel human-bovine chimeric PIV vaccine candidate.

Preferably, the degree of host range restriction exhibited by human-bovine
30 chimeric PIV vaccine candidates of the invention is comparable to the degree of host range restriction exhibited by the respective BPIV parent or "donor" strain. Preferably, the restriction should have a true host range phenotype, i.e., it should be specific to the host in question and should not restrict replication and vaccine preparation *in vitro* in a suitable cell line. In addition, human-bovine chimeric PIV bearing one or more bovine

gene(s) or genome segment(s) elicit a high level of resistance in hosts susceptible to PIV infection. Thus, the invention provides a new basis for attenuating a live virus vaccine against PIV, one which is based on host range effects due to the introduction of one or more gene(s) or genome segment(s) from a heterologous PIV, e.g., between HPIV3 and BPIV3.

In related aspects of the invention, human-bovine chimeric PIV incorporates one or more heterologous gene(s) that encode an HPIV HN and/or F glycoprotein(s). Alternatively, the chimeric PIV may incorporate one or more genome segment(s) encoding an ectodomain (and alternatively a cytoplasmic domain and/or transmembrane domain), or immunogenic epitope of an HPIV HN and/or F glycoprotein(s). These immunogenic proteins, domains and epitopes are particularly useful within human-bovine chimeric PIV because they generate novel immune responses in an immunized host. In particular, the HN and F proteins, and immunogenic domains and epitopes therein, provide major protective antigens.

In certain embodiments of the invention, addition or substitution of one or more immunogenic gene(s) or genome segment(s) from a human PIV subgroup or strain to or within a bovine background, or recipient, genome or antigenome yields a recombinant, chimeric virus or subviral particle capable of generating an immune response directed against the human donor virus, including one or more specific human PIV subgroups or strains, while the bovine backbone confers an attenuated phenotype making the chimera a useful candidate for vaccine development. In one exemplary embodiment, one or more human PIV glycoprotein genes, e.g., HN and/or F, are added to or substituted within a partial or complete bovine genome or antigenome to yield an attenuated, infectious human-bovine chimera that elicits an anti-human PIV immune response in a susceptible host.

In alternate embodiments, human-bovine chimeric PIV additionally incorporate a gene or genome segment encoding an immunogenic protein, protein domain or epitope from multiple human PIV strains, for example two HN or F proteins or immunogenic portions thereof each from a different HPIV, e.g., HPIV1 or HPIV2.

Alternatively, one glycoprotein or immunogenic determinant may be provided from a first HPIV, and a second glycoprotein or immunogenic determinant may be provided from a second HPIV by substitution without the addition of an extra glycoprotein- or determinant- encoding polynucleotide to the genome or antigenome. Substitution or addition of HPIV glycoproteins and antigenic determinants may also be achieved by

construction of a genome or antigenome that encodes a chimeric glycoprotein in the recombinant virus or subviral particle, for example having an immunogenic epitope, antigenic region or complete ectodomain of a first HPIV fused to a cytoplasmic domain of a heterologous HPIV. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a HPIV1 or HPIV2 HN or F glycoprotein may be joined with a genome segment encoding a corresponding HPIV3 HN or F glycoprotein cytoplasmic/endodomain in the background genome or antigenome.

In alternate embodiments a human-bovine chimeric PIV genome or antigenome may encode a substitute, extra, or chimeric glycoprotein or antigenic determinant thereof in the recombinant virus or subviral particle, to yield a viral recombinant having both human and bovine glycoproteins, glycoprotein domains, or immunogenic epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV HN or F glycoprotein may be joined with a genome segment encoding a corresponding bovine HN or F glycoprotein cytoplasmic/endodomain in the background genome or antigenome. Alternatively, the human PIV HN or F glycoprotein or parts thereof may be joined with a genome segment encoding an HN or F glycoprotein or parts thereof from another PIV strain or serotype.

Thus, according to the methods of the invention, human-bovine chimeric PIV may be constructed by substituting the heterologous gene or genome segment for a counterpart gene or genome segment in a partial PIV background genome or antigenome. Alternatively, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment in combination with a complete (or partial if another gene or genome segment is deleted) PIV background genome or antigenome. For example, two human PIV HN or F genes or genome segments can be included, one each from HPIV2 and HPIV3.

Often, a heterologous gene or genome segment is added near an intergenic position within a partial or complete PIV background genome or antigenome. Alternatively, the gene or genome segment can be placed in other noncoding regions of the genome, for example, within the 5' or 3' noncoding regions or in other positions where noncoding nucleotides occur within the partial or complete genome or antigenome. In one aspect, noncoding regulatory regions contain cis-acting signals required for efficient replication, transcription, and translation, and therefore represent target sites for modification of these functions by introducing a heterologous gene or genome segment or other mutation as disclosed herein.

In more detailed aspects of the invention, attenuating mutations are introduced into cis-acting regulatory regions to yield, e.g., (1) a tissue specific attenuation (Gromeier et al., J. Virol. 73:958-964, 1999; Zimmermann et al., J. Virol. 71:4145-4149, 1997), (2) increased sensitivity to interferon (Zimmermann et al., 1997, *supra*), (3) 5 temperature sensitivity (Whitehead et al., 1998a, *supra*), (4) a general restriction in level of replication (Men et al., J. Virol. 70:3930-3937, 1996; Muster et al., Proc. Natl. Acad. Sci. USA 88:5177-5181, 1991), and/or (5) host specific restriction of replication (Cahour et al., Virology 207:68-76, 1995). These attenuating mutations can be achieved in various ways to produce an attenuated human-bovine chimeric PIV of the invention, for 10 example by point mutations, swaps of sequences between related viruses, or nucleotide deletions.

In yet additional alternative methods provided herein, a heterologous gene or genome segment may be added or substituted at a position corresponding to a wild-type gene order position of a counterpart gene or genome segment within the partial or 15 complete PIV background genome or antigenome. In other embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the background genome or antigenome, to enhance or reduce expression, respectively, of the heterologous gene or genome segment.

20 In general aspects of the invention, bovine genes or genome segments may be added to or substituted within a human PIV background to form an attenuated, human-bovine chimeric PIV. Alternatively, the chimera may be comprised of one or more human gene(s) or genome segment(s) added to or substituted within a bovine PIV background to form an attenuated PIV vaccine candidate. In this context, a chimeric PIV 25 genome or antigenome is formed of a partial or complete bovine PIV background genome or antigenome combined with a heterologous gene or genome segment from a human PIV. In preferred aspects, one or more bovine PIV gene(s) or genome segment(s) is substituted for a counterpart gene(s) or genome segment(s) within a human PIV background genome or antigenome. In alternate embodiments, one or more human PIV 30 glycoprotein genes, e.g., HN and/or F or a genome segment encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of a human PIV glycoprotein gene is substituted for a counterpart gene or genome segment within the bovine PIV background genome or antigenome. For example, both human PIV

glycoprotein genes HN and F may be substituted to replace counterpart HN and F glycoprotein genes in a bovine PIV background genome or antigenome.

In a parallel fashion, the chimeric human-bovine PIV of the invention can be readily designed as "vectors" to incorporate antigenic determinants from different pathogens, including more than one PIV strain or group (e.g., both human PIV3 and human PIV1), respiratory syncytial virus (RSV), measles and other pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference).

In more detailed aspects of the invention, human-bovine chimeric PIV are comprised of a partial or complete BPIV background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV. Within these aspects, one or more of the HPIV glycoprotein genes HN and F, or one or more genome segments encoding a cytoplasmic domain, transmembrane domain, ectodomain or immunogenic epitope of the HN and/or F genes, may be added to a BPIV background genome or antigenome or substituted for one or more counterpart genes or genome segments within the BPIV background genome or antigenome to yield the chimeric construct. Often, both HPIV glycoprotein genes HN and F will be substituted to replace counterpart HN and F glycoprotein genes in the BPIV background genome or antigenome, as exemplified by the recombinant chimeric virus rBPIV3-F_HHN_H described below. This is a desirable construct because it combines the antigenic determinants of the human PIV with the host range restricting elements of the bovine PIV.

In combination with the host range phenotypic effects provided in the human-bovine chimeric PIV of the invention, it is often desirable to adjust the attenuation phenotype by introducing additional mutations that increase or decrease attenuation of the chimeric virus. Thus, in additional aspects of the invention, attenuated, human-bovine chimeric PIV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or subviral particle. These can include mutations in RNA regulatory sequences or in encoded proteins. These attenuating mutations may be generated *de novo* and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant PIV and thereafter incorporated into a human-bovine chimeric PIV of the invention.

Introduction of attenuating and other desired phenotype-specifying mutations into chimeric bovine-human PIV of the invention may be achieved by transferring a heterologous gene or genome segment, e.g., a gene encoding an L protein or portion thereof, into a bovine or human PIV background genome or antigenome.

5 Alternatively, the mutation may be present in the selected background genome or antigenome, and the introduced heterologous gene or genome segment may bear no mutations or may bear one or more different mutations. Typically, the human or bovine background or "recipient" genome or antigenome is modified at one or more sites corresponding to a site of mutation in a heterologous virus (e.g., a heterologous bovine or
10 human PIV or a non-PIV negative stranded RNA virus) to contain or encode the same or a conservatively related mutation (e.g., a conservative amino acid substitution) as that identified in the donor virus (see, PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference). In one exemplary embodiment, a bovine background
15 or "recipient" genome or antigenome is modified at one or more sites corresponding to a site of mutation in HPIV3 JS *cp45*, as enumerated below, to contain or encode the same or a conservatively related mutation as that identified in the *cp45* "donor."

Preferred mutant PIV strains for identifying and incorporating attenuating mutations into bovine-human chimeric PIV of the invention include cold passaged (cp),
20 cold adapted (ca), host range restricted (hr), small plaque (sp), and/or temperature sensitive (ts) mutants, for example the JS HPIV3 cp 45 mutant strain. In exemplary embodiments, one or more attenuating mutations occur in the polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS wild type HPIV3. Alternatively, attenuating mutations in the N protein may be selected and incorporated in
25 a human-bovine chimeric PIV, for example which encode amino acid substitution(s) at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS. Alternative or additional mutations may encode amino acid substitution(s) in the C protein, e.g., at a position corresponding to Ile₉₆ of JS and in the M protein, e.g., at a position corresponding to Pro₁₉₉ (for example a Pro₁₉₉ to Thr mutation). Yet additional mutations for adjusting
30 attenuation of a human-bovine chimeric PIV of the invention are found in the F protein, e.g., at a position corresponding to Ile₄₂₀ or Ala₄₅₀ of JS, and in the HN protein, e.g., at a position corresponding to residue Val₃₈₄ of JS.

Attenuating mutations from biologically derived PIV mutants for incorporation into human-bovine chimeric PIV of the invention also include mutations in

noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS cp45. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS cp45.

From JS cp45 and other biologically derived PIV and non-PIV mutants, a large "menu" of attenuating mutations is provided, each of which mutations can be combined with any other mutation(s) for adjusting the level of attenuation in a recombinant PIV bearing a genome or antigenome that is a chimera of human and bovine gene(s) or genome segment(s). For example, mutations within recombinant PIV of the invention include one or more, and preferably two or more, mutations of JS cp45. Desired human-bovine chimeric PIV of the invention selected for vaccine use often have at least two and sometimes three or more attenuating mutations to achieve a satisfactory level of attenuation for broad clinical use. Preferably, recombinant human-bovine chimeric PIV incorporate one or more attenuating mutation(s) stabilized by multiple nucleotide substitutions in a codon specifying the mutation.

Additional mutations which can be adopted or transferred to human-bovine chimeric PIV of the invention may be identified in non-PIV nonsegmented negative stranded RNA viruses and incorporated in PIV mutants of the invention. This is readily accomplished by mapping the mutation identified in a heterologous negative stranded RNA virus to a corresponding, homologous site in a recipient PIV genome or antigenome and mutating the existing sequence in the recipient to the mutant genotype (either by an identical or conservative mutation), as described in PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference.

In addition to recombinant human-bovine chimeric PIV, the invention provides related cDNA clones, vectors and particles, each of which incorporate HPIV and BPIV sequences and, optionally, one or more of the additional, phenotype-specific mutations set forth herein. These are introduced in selected combinations, e.g., into an isolated polynucleotide which is a recombinant cDNA genome or antigenome, to produce a suitably attenuated, infectious virus or subviral particle upon expression, according to the methods described herein. This process, coupled with routine phenotypic evaluation, provides human-bovine chimeric PIV having such desired characteristics as attenuation,

temperature sensitivity, altered immunogenicity, cold-adaptation, small plaque size, host range restriction, genetic stability, etc. In particular, vaccine candidates are selected which are attenuated and yet are sufficiently immunogenic to elicit a protective immune response in the vaccinated mammalian host.

5 In yet additional aspects of the invention, human-bovine chimeric PIV, with or without additional mutations adopted, e.g., from a biologically derived attenuated mutant virus, are constructed to have additional nucleotide modification(s) to yield a desired phenotypic, structural, or functional change. Typically, the selected nucleotide modification will specify a phenotypic change, for example a change in growth
10 characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host range restriction, or immunogenicity. Structural changes in this context include introduction or ablation of restriction sites into PIV encoding cDNAs for ease of manipulation and identification.

In preferred embodiments, nucleotide changes within the genome or
15 antigenome of a human-bovine chimeric PIV include modification of a viral gene by partial or complete deletion of the gene or reduction or ablation (knock-out) of its expression. These modifications can be introduced within the human or bovine background genome or antigenome, or may be introduced into the chimeric genome or antigenome by incorporation within the heterologous gene(s) or genome segment(s)
20 added or substituted therein. Target genes for mutation in this context include any of the PIV genes, including the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, small hydrophobic SH protein, where applicable, fusion protein F, and the products of the C, D and V open reading frames (ORFs). To the extent that the recombinant virus remains
25 viable and infectious, each of these proteins can be selectively deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to achieve novel deletion or knock out mutants. For example, one or more of the C, D, and/or V genes may be deleted in whole or in part, or its expression reduced or ablated (e.g., by introduction of a stop codon, by a mutation in an RNA editing site, by a mutation
30 that alters the amino acid specified by an initiation codon, or by a frame shift mutation in the targeted ORF(s). In one embodiment, a mutation can be made in the editing site that prevents editing and ablates expression of proteins whose mRNA is generated by RNA editing (Kato et al., EMBO J. 16:578-587, 1997a and Schneider et al., Virology 227:314-322, 1997, each incorporated herein by reference). Alternatively, one or more of the C,

D, and/or V ORF(s) can be deleted in whole or in part to alter the phenotype of the resultant recombinant clone to improve growth, attenuation, immunogenicity or other desired phenotypic characteristics (see, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference).

5 Alternative nucleotide modifications in human-bovine chimeric PIV of the invention include a deletion, insertion, addition or rearrangement of a cis-acting regulatory sequence for a selected gene in the recombinant genome or antigenome. As with other such modifications described herein, these modifications can be introduced within the human or bovine background genome or antigenome, or may be introduced
10 into the chimeric genome or antigenome by incorporation within the heterologous gene(s) or genome segment(s) added or substituted therein. In one example, a cis-acting regulatory sequence of one PIV gene is changed to correspond to a heterologous regulatory sequence, which may be a counterpart cis-acting regulatory sequence of the same gene in a different PIV, or a cis-acting regulatory sequence of a different PIV gene.
15 For example, a gene end signal may be modified by conversion or substitution to a gene end signal of a different gene in the same PIV strain. In other embodiments, the nucleotide modification may comprise an insertion, deletion, substitution, or rearrangement of a translational start site within the recombinant genome or antigenome, e.g., to ablate an alternative translational start site for a selected form of a protein.

20 In addition, a variety of other genetic alterations can be produced in a human-bovine chimeric PIV genome or antigenome, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV. For example, genes or genome segments from non-PIV sources may be inserted in whole or in part. Alternatively, the order of genes can be changed, or a PIV genome promoter replaced
25 with its antigenome counterpart. Different or additional modifications in the recombinant genome or antigenome can be made to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

30 In yet additional aspects, polynucleotide molecules or vectors encoding the human-bovine chimeric PIV genome or antigenome can be modified to encode non-PIV sequences, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein or immunogenic epitope of a microbial pathogen (e.g., virus, bacterium, parasite, or fungus) capable of eliciting a protective immune response in an intended host. In one such embodiment, human-bovine chimeric PIV are constructed that incorporate a gene or

genome segment from a respiratory syncytial virus (RSV), for example a gene encoding an antigenic protein (e.g., an F or G protein), immunogenic domain or epitope of RSV.

In related aspects of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating a PIV-encoding cDNA) and methods are provided for producing an isolated infectious human-bovine chimeric PIV. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a human-bovine chimeric PIV genome or antigenome. Also provided is the same or different expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins. These proteins also can be expressed directly from the genome or antigenome cDNA. The vector(s) is/are preferably expressed or coexpressed in a cell or cell-free lysate, thereby producing an infectious human-bovine chimeric PIV viral particle or subviral particle.

The above methods and compositions for producing human-bovine chimeric PIV yield infectious viral or subviral particles, or derivatives thereof. A recombinant infectious virus is comparable to the authentic PIV virus particle and is infectious as is. It can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA and the N, P, and L proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided within the invention include viral particles which lack one or more protein(s), protein segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell-free lysate containing an expression vector which comprises an isolated polynucleotide molecule comprising a human-bovine chimeric PIV genome or antigenome as described above, and an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, and L proteins of PIV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression the genome or antigenome and N, P and L combine to produce an infectious human-bovine chimeric PIV virus or subviral particle.

The human-bovine chimeric PIVs of the invention are useful in various compositions to generate a desired immune response against PIV in a host susceptible to PIV infection. Human-bovine chimeric PIV recombinants are capable of eliciting a

protective immune response in an infected mammalian host, yet are sufficiently attenuated so as not to cause unacceptable symptoms of severe respiratory disease in the immunized host. In addition, the human-bovine chimeric PIV recombinants should replicate with sufficient efficiency *in vitro* to make vaccine preparation feasible. The attenuated virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

The invention further provides novel vaccines comprising a physiologically acceptable carrier and/or adjuvant and an isolated attenuated human-bovine chimeric PIV virus or subviral particle. In preferred embodiments, the vaccine is comprised of a human-bovine chimeric PIV having at least one, and preferably two or more additional mutations or other nucleotide modifications as described above to achieve a suitable balance of attenuation and immunogenicity. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine may comprise attenuated human-bovine chimeric PIV that elicits an immune response against a single PIV strain or against multiple PIV strains or groups. In this regard, human-bovine chimeric PIV can be combined in vaccine formulations with other PIV vaccine strains, or with other viral vaccine viruses such as RSV.

In related aspects, the invention provides a method for stimulating the immune system to elicit an immune response against PIV in a mammalian subject. The method comprises administering a formulation of an immunologically sufficient amount of a human-bovine chimeric PIV in a physiologically acceptable carrier and/or adjuvant. In one embodiment, the immunogenic composition is a vaccine comprised of a human-bovine chimeric PIV having at least one, and preferably two or more attenuating mutations or other nucleotide modifications specifying a desired phenotype as described above. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine may comprise attenuated human-bovine chimeric PIV virus that elicits an immune response against a single PIV, against multiple PIVs, e.g., HPIV1 and HPIV3, or against one or more PIV(s) and a non-PIV pathogen such as RSV. In this context, human-bovine chimeric PIV can elicit a monospecific immune response or a polyspecific immune response against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen such as RSV. Alternatively, human-bovine chimeric PIV having different immunogenic characteristics can be combined in a vaccine mixture or administered

separately in a coordinated treatment protocol to elicit more effective protection against one PIV, against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen such as RSV. Preferably the immunogenic composition is administered to the upper respiratory tract, e.g., by spray, droplet or aerosol.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates cloning of the N coding region of bovine PIV strains Ka or SF into HPIV3. In Fig. 1A-C, the BPIV3 N open reading frame (ORF) replaces its
10 corresponding HPIV3 sequence in the full-length rJS antigenomic cDNA (Durbin et al., 1997a, *supra*). BPIV3 Ka and SF N genes were first amplified by RT-PCR using standard molecular biological techniques from virion RNA and subcloned as 1.9 kb fragments into pBluescript to give pBS-KaN or pBS-SFN, respectively. The HPIV3 rJS N gene was subcloned as a 1.9 kb MluI/EcoRI fragment into pUC 119 from a plasmid
15 containing the 5' half of the rJS HPIV3 antigenome (Durbin et al., 1997a, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed
20 September 19, 1997, each incorporated herein by reference) to give pUC119JSN. Each N gene was modified by site-directed mutagenesis to place an NcoI and AflII site at the translational start and stop sites, respectively. The Ka and SF N genes are identical in the translational start and stop site regions and, therefore, identical mutagenesis reactions were performed on both BPIV3 N genes as depicted in 1A. Fig. 1B--Following AflII/NcoI digestion, a 1.5 kb fragment from pBS-KaN or pBS-SFN representing the
25 BPIV3 N coding region was introduced into the NcoI/AflII window of the HPIV3 N subclone pUC119JSN-NcoI/AflII as a replacement for its HPIV3 counterpart. Fig. 1C-- Each chimeric subclone was then subjected to site-directed mutagenesis to restore the sequence present in HPIV3 rJS before the translation start codon or after the stop codon and BPIV3 coding sequence immediately after the start codon and before the stop codon.
30 This yielded pUC119B/HKaN and pUC119B/HSFN, which were used to import the BPIV3 N gene into the HPIV3 cDNA clone as shown in Fig. 2.

Figure 2 illustrates insertion of the HPIV3/BPIV3 (strain Ka or SF) chimeric N gene into the HPIV3 antigenomic cDNA. In Fig. 2A, the BPIV3 N ORF of Ka or SF flanked by HPIV3 sequence was subcloned as an MluI/EcoRI fragment from

pUC119B/HKaN or pUC119B/HSFN and inserted into pLeft+2G (Durbin et al., 1997a, *supra*). The pLeft+2G plasmid contains the 5' half of the HPIV3 rJS antigenome from nt 1-7437 (genome sense) behind a T7 promoter. The location of two G residues that were inserted between T7 promoter and HPIV3 sequence to improve transcription is indicated by an asterisk. Fig. 2B--An XhoI/NgoMI fragment of pRight (Durbin et al., 1997a, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference) containing the 3' end of the HPIV3 antigenome flanked by the hepatitis delta virus ribozyme and T7 terminator was cloned into the XhoI/NgoMI window of the modified pLeft plasmid resulting in plasmids pB/HPIV3KaN and pB/HPIV3SFN. Each of these chimeric constructs contains the complete positive sense sequence of the HPIV3 antigenomic RNA except for the N coding region which has been replaced by its BPIV3 Ka or SF counterpart.

Figure 3 provides nucleotide sequences of HPIV3, BPIV3 and chimeric viruses of the invention around N translation start (A) and stop (B) codons. The position of the individual ORFs is described in the respective Genbank reports (#AF178654 for BPIV3 Ka, #AF178655 for BPIV3 SF and #Z11515) and included herein by reference. The sequences (positive-sense) flanking the translational start (A) and stop (B) codons (each underlined) in the N gene are shown for the parental recombinant HPIV3 JS (rJS), the parental biologically-derived BPIV3 Ka and SF viruses (Ka and SF), and the chimeric cKa and cSF viruses. Host-specific residues in the cKa and cSF virus sequences and their counterparts in rJS (before the start codon and after the stop codon) and SF or Ka (start codon through stop codon, inclusive) are in boldface type. Plaque-purified chimeric virus was amplified by RT-PCR from virion RNA and sequenced using the Taq Dye Deoxy Terminator Cycle kit (ABI, Foster City, CA). This confirmed that the predicted sequences were present in each chimeric virus.

Figure 4 details the structure of the BPIV3/HPIV3 chimeric viruses of the invention, and their confirmation by TaqI digestion of RT-PCR products generated from virus RNA. In Fig. 4A the genomes of the chimeric cKa and cSF viruses are shown schematically (not to scale) relative to that of HPIV3 and BPIV3 parent viruses. Ka- and SF-specific regions are indicated by light and dark shading respectively. Arrows above the rJS genome indicate the locations of primers used for RT-PCR amplification of chimeric and parent viruses for the purposes of diagnostic TaqI digestion. These primers

were directed to regions conserved between HPIV3 and BPIV3 so that they could be used for the amplification of HPIV3, BPIV3 and chimeric BPIV3/HPIV3 viruses. In Fig. 4B the expected sizes of TaqI digestion products for each virus are shown for a 1898-bp PCR product amplified from RNA with the primer pair illustrated in Fig. 4A. This PCR product is illustrated at the top in Fig. 4B, and the N ORF is indicated as a filled rectangle. TaqI fragments unique to each virus and which therefore serve in virus identification are indicated with an asterisk. Fig. 4C provides TaqI profiles of PCR products containing the PIV3 N coding region of chimeric cKa (left) or cSF (right) flanked by those of the HPIV3 and BPIV3 parent viruses. Unique TaqI fragments diagnostic of virus identity and corresponding to those identified in (4B) are indicated with an asterisk. Calculated lengths (bp) of DNA gel bands are indicated.

Figure 5 provides multicycle growth curves of parental and chimeric viruses in MDBK (A) or LLC-MK2 (B) cells. Monolayers of bovine MDBK (A) or simian LLC-MK2 (B) cells in wells (9.6 cm² each) of a 6 well plate were infected individually at a multiplicity of infection of 0.01 with the indicated parental or chimeric virus. Three replicate infections were performed for each virus. Samples were taken at the indicated time points, stored at -70°C, and titered by TCID₅₀ assay in parallel. Growth curves are constructed using the average of 3 replicate samples at each time point. The lower limit of virus detectability was 10^{1.5}TCID₅₀/ml, which is indicated by a dotted line.

Figures 6A-6G set forth the complete positive sense nucleotide sequence of the bovine PIV3 Ka strain.

Figures 7A-7G set forth the complete positive sense nucleotide sequence of the bovine PIV3 SF strain.

Figure 8A provides a schematic depiction of the genomes of chimeric rHPIV3-F_BHN_B and rBPIV3-F_HHN_H viruses, and of their parent viruses, rHPIV3 JS and BPIV3 Ka (not to scale). The F and HN genes were exchanged in a single restriction fragment between rHPIV3 and rBPIV3 using *Sgr*AI and *Bsi*WI sites that had been introduced in front of the M and HN gene end sequences, respectively.

Figure 8B depicts assembly of an antigenomic cDNA for BPIV3 Ka. A full length cDNA was constructed to encode the complete antigenomic sequence of BPIV3 Ka (GenBank accession #AF178654). The cDNA was assembled from subclones derived from reverse transcription (RT) of viral (v)RNA and polymerase chain reaction

(PCR) amplification. Multiple subclones of the antigenome were sequenced, and only clones matching the consensus sequence of BPIV3 Ka were used for assembly of the full length clone, with the exception of nt 21 and nt 23, which differ from the published sequence but occur with similar frequency in the virus population..

5 Figure 8C illustrates features of parental and chimeric bovine-human PIV genomes. The genomes of the chimeric rHPIV3 F_BHN_B and rBPIV3 F_HHN_H viruses and those of their parent viruses rHPIV3 JS and BPIV3 Ka are shown schematically (not to scale). Two unique restriction enzyme recognition sites, SgrAI and BsiWI, were introduced near the M and HN gene ends, respectively. The recombinant HPIV3 and
10 BPIV3 viruses bearing these introduced restriction sites were designated rHPIV3s and rBPIV3s as indicated in Figure 8C2. Glycoprotein genes were exchanged between rHPIV3 JS and rBPIV3 Ka. The nucleotide sequence that was mutagenized is shown below each cDNA construct, with the position of the first nucleotide of each sequence indicated. The introduced SgrAI and BsiWI restriction sites are underlined and
15 nucleotides that differ between HPIV3 and BPIV3 and thus identify the origin of the gene inserts are depicted in bold print.

 Figure 9 provides a confirmation of the identity of recombinant viruses by RT-PCR of viral RNA and *Eco* RI digestion. RT-PCR products of viral RNA were prepared with a primer pair that recognized conserved regions on either side of the F and
20 HN genes in both BPIV3 and HPIV3. Digestion with *Eco* RI resulted in a unique pattern of restriction fragments for each of the four viruses. In the schematic diagram on the left, horizontal lines symbolize the amplified viral sequences and vertical bars show the positions of *Eco* RI sites. The expected size of each restriction fragment is indicated above the line. The numbers below each line correspond to the sequence position in the antigenomic RNA of BPIV3 Ka, HPIV3 JS (GenBank accession #AF178654 and
25 Z11575), or of the indicated chimeric derivative. On the right, a 1% agarose gel of the *Eco* RI digestion of PCR products is shown, confirming the identity of parental and chimeric viruses. The asterisks indicate gel bands that contain two restriction fragments that comigrate due to close similarity in size.

30 Figure 10 depicts multicycle replication of chimeric and parental viruses in simian LLC-MK2 cells. Multicycle replication (the input inoculum had an MOI of 0.01) of the three chimeric viruses rHPIV3-F_BHN_B, rBPIV3-F_HHN_H and rHPIV3-N_B (also referred to as cKa) is compared with the replication of their parental viruses BPIV3 Ka and rHPIV3. The virus titers are shown as mean log₁₀ TCID₅₀/ml ± standard error of

triplicate samples. The lower limit of detection of this assay is 10 TCID₅₀, as indicated by the dotted horizontal line.

Figure 11 documents mean titers of chimeric and parental viruses in nasopharyngeal swabs of infected rhesus monkeys over the course of infection. Virus titers are shown as mean TCID₅₀/ml in LLC-MK2 cells \pm standard error for groups of 4 or 6 monkeys infected with the same virus. This illustrates the same experiment as shown in Table 3. In panel A, mean titers of rHPIV3-F_BHN_B are compared to rHPIV3 and BPIV3 Ka titers. In panel B, mean rBPIV3-F_HHN_H titers are compared to those of BPIV3 Ka and rHPIV3, which, for the last two viruses, are the same values in panel A but are presented separately to facilitate comparison. Day 5 titers were excluded from the figures because they were much lower than day 4 and day 6 titers, most likely due to technical problems during the sample collection.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The present invention provides recombinant parainfluenza virus (PIV) cloned as a chimera of human and bovine PIV genomic or antigenomic sequences to yield a human-bovine chimeric PIV. The chimeric construction of human-bovine PIV yields a viral particle or subviral particle that is infectious in mammals, particularly humans, and useful for generating immunogenic compositions for clinical or veterinary use. Also provided within the invention are novel methods and compositions for designing and producing attenuated, human-bovine chimeric PIV, as well as methods and compositions for the prophylaxis and treatment of PIV infection.

Chimeric human-bovine PIV of the invention are recombinantly engineered to incorporate nucleotide sequences from both human and bovine PIV strains to produce an infectious, chimeric virus or subviral particle. In this manner, candidate vaccine viruses are recombinantly engineered to elicit an immune response against PIV in a mammalian host susceptible to PIV infection, including humans and non-human primates. Human-bovine chimeric PIV according to the invention may elicit an immune response to a specific PIV, e.g., HPIV3, or a polyspecific response against multiple PIVs, e.g., HPIV1 and HPIV3.

Exemplary human-bovine chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome comprising both human and bovine polynucleotide sequences, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein

(P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

5 Chimeric human-bovine PIV of the invention include a partial or complete “background” PIV genome or antigenome derived from or patterned after a human or bovine PIV strain or serotype virus combined with one or more heterologous gene(s) or genome segment(s) of a different PIV strain or serotype virus to form the human-bovine chimeric PIV genome or antigenome. In certain aspects of the invention, chimeric PIV
10 incorporate a partial or complete human PIV (HPIV) background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a bovine PIV. In alternate aspects of the invention, chimeric PIV incorporate a partial or complete bovine PIV (BPIV) background genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a human PIV.

15 The partial or complete background genome or antigenome typically acts as a recipient backbone or vector into which are imported heterologous genes or genome segments of the counterpart, human or bovine PIV. Heterologous genes or genome segments from the counterpart, human or bovine PIV represent “donor” genes or polynucleotides that are combined with, or substituted within, the background genome or
20 antigenome to yield a human-bovine chimeric PIV that exhibits novel phenotypic characteristics compared to one or both of the contributing PIVs. For example, addition or substitution of heterologous genes or genome segments within a selected recipient PIV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity, or other desired phenotypic changes as compared with a corresponding
25 phenotype(s) of the unmodified recipient and/or donor. Genes and genome segments that may be selected for use as heterologous inserts or additions within human-bovine chimeric PIV of the invention include genes or genome segments encoding a PIV N, P, C, D, V, M, SH, where applicable, F, HN and/or L protein(s) or portion(s) thereof. Regulatory regions, such as the extragenic leader or trailer or intergenic regions, are also
30 useful as heterologous inserts or additions.

The heterologous gene(s) or genome segment(s) may be added or substituted at a position corresponding to a wild-type gene order position of the counterpart gene(s) or genome segment(s) within the partial or complete PIV background genome or antigenome, which counterpart gene or genome segment is thereby replaced or

displaced (e.g., to a more promotor-distal position). In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promotor-distal compared to a wild-type gene order position of the counterpart gene or genome segment within the background genome or antigenome, which enhances or reduces, respectively, expression of the heterologous gene or genome segment.

The introduction of heterologous immunogenic proteins, domains and epitopes to produce human-bovine chimeric PIV is particularly useful to generate novel immune responses in an immunized host. Addition or substitution of an immunogenic gene or genome segment from one, donor PIV within a recipient genome or antigenome of a different PIV can generate an immune response directed against the donor subgroup or strain, the recipient subgroup or strain, or against both the donor and recipient subgroup or strain. To achieve this purpose, human-bovine chimeric PIV may also be constructed that express a chimeric protein, e.g., an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to one PIV fused to an ectodomain of a different PIV to provide, e.g., a human-bovine fusion protein, or a fusion protein incorporating domains from two different human PIVs. In a preferred embodiment, a human-bovine chimeric PIV genome or antigenome encodes a chimeric glycoprotein in the recombinant virus or subviral particle having both human and bovine glycoprotein domains or immunogenic epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV HN or F glycoprotein may be joined with a polynucleotide sequence (i.e., a genome segment) encoding the corresponding bovine HN or F glycoprotein cytoplasmic and transmembrane domains to form the human-bovine chimeric PIV genome or antigenome.

In other embodiments, human-bovine chimeric PIV useful in a vaccine formulation can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the HN and/or F proteins. This might involve the introduction of one or more point mutations; it might also involve an entire HN or F gene, or a genome segment encoding a particular immunogenic region thereof, from one PIV strain or group is incorporated into a chimeric PIV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different PIV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented. Progeny virus produced from the modified PIV clone can then be used in vaccination protocols against emerging PIV strains.

Replacement of a human PIV coding sequence or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a heterologous counterpart yields chimeric PIV having a variety of possible attenuating and other phenotypic effects. In particular, host range and other desired effects arise from substituting a bovine or murine PIV (MPIV) protein, protein domain, gene or genome segment imported within a human PIV background, wherein the bovine or murine gene does not function efficiently in a human cell, e.g., from incompatibility of the heterologous sequence or protein with a biologically interactive human PIV sequence or protein (i.e., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, bovine PIV sequences are selected for introduction into human PIV based on known aspects of bovine and human PIV structure and function.

HPIV3 is a member of the *Respirovirus* genus of the *Paramyxoviridae* family in the order *Mononegavirales* (Collins et al., 1996, *supra*). HPIV3 is the best characterized of the HPIVs and represents the prototype HPIV. Its genome is a single strand of negative-sense RNA 15462 nucleotides (nt) in length (Galinski et al., *Virology* 165:499-510, 1988; and Stokes et al., *Virus Res.* 25:91-103, 1992; each incorporated herein by reference). At least eight proteins are encoded by the PIV3 genome: the nucleocapsid protein N, the phosphoprotein P, the C and D proteins of unknown functions, the matrix protein M, the fusion glycoprotein F, the hemagglutinin-neuraminidase glycoprotein HN, and the large polymerase protein L (Collins et al., 1996, *supra*). A protein containing the V ORF in the P gene might also be produced (Durbin et al., *Virology* 261:319-333, 1999)

The M, HN, and F proteins are envelope-associated, and the latter two are surface glycoproteins which, as is the case with each PIV, are the major neutralization and protective antigens (Collins et al., 1996, *supra*). The significant sequence divergence between comparable PIV HN or F proteins among the PIVs is thought to be the basis for the type specificity of the protective immunity (Collins et al., 1996, *supra*; Cook et al., *Amer. Jour. Hyg.* 77:150-159, 1963; Ray et al., *J. Infect. Dis.* 162:746-749, 1990; each incorporated herein by reference).

The HPIV3 genes are each transcribed as a single mRNA that encodes a single protein, with the exception of the P mRNA which contains four ORFs, namely P,

C, D and V (Galinski et al., Virology 186:543-550, 1992; and Spriggs et al., J. Gen. Virol. 67:2705-2719, 1986; each incorporated herein by reference). The P and C proteins are translated from separate, overlapping ORFs in the mRNA. Whereas all paramyxoviruses encode a P protein, only members of the genus *Respirovirus* and *Morbillivirus* encode a C protein. Individual viruses vary in the number of proteins expressed from the C ORF and in its importance in replication of the virus *in vitro* and *in vivo*. Sendai virus (SeV) expresses four independently initiated proteins from the C ORF: C', C, Y1, and Y2, whose translational start sites appear in that order in the mRNA (Curran, et al., Enzyme 44:244-249, 1990; Lamb et al., in The Paramyxoviruses, D. Kingsbury, ed., pp. 181-214, Plenum Press, New York, 1991; incorporated herein by reference), whereas HPIV3 and measles virus (MeV) express only a single C protein (Bellini et al., J. Virol. 53:908-919, 1985; Sanchez et al., Virology 147:177-86, 1985; and Spriggs et al., 1986, *supra*; each incorporated herein by reference).

The PIV3 D protein is a fusion protein of the P and D ORFs, and is expressed from the P gene by the process of co-transcriptional RNA editing in which two nontemplated G residues are added to the P mRNA at the RNA editing site (Galinski et al., 1992, *supra*; and Pelet et al., EMBO J. 10:443-448, 1991; each incorporated herein by reference). BPIV3, the only other paramyxovirus which expresses a D protein, uses RNA editing to express this protein as well as a second protein, the V protein.

Nearly all members of the genera *Respirovirus*, *Rubulavirus*, and *Morbillivirus* express a V protein. The one member which clearly does not is HPIV1, which lacks an intact V ORF (Matsuoka et al., J. Virol. 65:3406-3410, 1991, incorporated herein by reference). The V ORF is characterized by the presence of a cysteine-rich domain that is highly conserved (Cattaneo et al., Cell 56:759-764, 1989; Park et al., J. Virol. 66:7033-7039, 1992; Thomas et al., Cell 54:891-902, 1988; and Vidal et al., J. Virol. 64:239-246, 1990; each incorporated herein by reference). The V ORF is maintained in each of the HPIV3 viruses sequenced to date suggesting that this ORF is expressed and retains function for this virus (Galinski et al., Virology 155:46-60, 1986; Spriggs et al., 1986, *supra*; and Stokes et al., 1992, *supra*; incorporated herein by reference).

The BPIV3 V protein is expressed when one nontemplated G residue is added at the RNA editing site (Pelet et al., 1991, *supra*; incorporated herein by reference). However, in the case of HPIV3, two translation stop codons lie between the editing site and the V ORF, and it is not clear whether HPIV3 represents another example in which

this ORF is not expressed, or whether it is expressed by some other mechanism. One possibility is that HPIV3 editing also occurs at a second, downstream site in the P gene, although this did not appear to occur in cell culture (Galinski et al., 1992, *supra*).

Alternatively, it might be that ribosomes gain access to the V ORF by ribosomal

5 frameshifting. This would be comparable to the situation with the P locus of MV. MV expresses C, P, and V proteins, but also expresses a novel R protein which is synthesized by frameshifting from the P ORF to the V ORF (Liston et al., *J. Virol.* 69:6742-6750, 1995, incorporated herein by reference). Genetic evidence suggests that the V ORF of HPIV3 is functional (Durbin et al., 1999, *supra*).

10 Although the means by which HPIV3 expresses its V protein is unclear, the extreme conservation of the its V ORF in different strains suggests that it is indeed expressed. The function of the V protein is not well defined, but V-minus MV and SeV recombinants have been recovered that replicate efficiently *in vitro* but exhibit reduced replication *in vivo* (Delenda, et al., *Virology* 228:55-62, 1997; Delenda et al., *Virology* 242:327-337, 1998; Kato et al., 1997a, *supra*; Kato et al., *J. Virol.* 71:7266-7272, 1997b; 15 and Valsamakis et al., *J. Virol.* 72:7754-7761, 1998; each incorporated herein by reference).

The viral genome of PIV also contains extragenic leader and trailer regions, possessing all or part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Thus, the PIV genetic map is represented as 3' leader-N-P/C/D/V-M-F-HN-L-5' trailer. Some viruses, such as simian virus 5 and mumps virus, have a gene located between F and HN that encodes a small hydrophobic (SH) protein of unknown function. Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs 20 found at the gene boundaries. The upstream end of each gene contains a gene-start (GS) signal, which directs initiation of its respective mRNA. The downstream terminus of each gene contains a gene-end (GE) motif which directs polyadenylation and termination. Exemplary sequences have been described for the human PIV3 strains JS (GenBank accession number Z11575, incorporated herein by reference) and Washington (Galinski 25 M.S., in *The Paramyxoviruses*, Kingsbury, D.W., ed., pp. 537-568, Plenum Press, New York, 1991, incorporated herein by reference), and for the bovine PIV3 strain 910N (GenBank accession number D80487, incorporated herein by reference).

As used herein, "PIV gene" generally refers to a portion of the PIV genome encoding an mRNA and typically begins at the upstream end with a gene-start

(GS) signal and ends at the downstream end with the gene-end (GE) signal. The term PIV gene also includes what is described as “translational open reading frame”, or ORF, particularly in the case where a protein, such as C, is expressed from an additional ORF rather than from a unique mRNA. To construct human-bovine chimeric PIV of the invention, one or more PIV gene(s) or genome segment(s) may be deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include open reading frames and/or cis-acting regulatory sequences of any one or more of the PIV genes or genome segments. By “genome segment” is meant any length of continuous nucleotides from the PIV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof.

The instant invention involves a method for developing live attenuated PIV vaccine candidates based on chimeras between HPIVs and BPIV3. Chimeras are constructed through a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and are propagated in the same manner as if they were biologically-derived viruses. Preferred human-bovine chimeric PIV vaccine candidates of the invention bear one or more of the major antigenic determinants of one or more human PIV(s), e.g., HPIV1, HPIV2, and/or HPIV3, in a background which is attenuated by the substitution or addition of one or more BPIV genes or genome segments. The major protective antigens of PIVs are their HN and F glycoproteins, although other proteins can also contribute to a protective immune response.

Thus, the invention provides a new basis for attenuating a wild type or mutant parental virus for use as a vaccine against PIV, one which is based on host range effects due to the introduction of one or more gene(s) or genome segment(s) between HPIV and BPIV. There are numerous nucleotide and amino acid sequence differences between BPIV and HPIV, which are reflected in host range differences. For example, between HPIV3 and BPIV3 the percent amino acid identity for each of the following proteins is: N (86%), P (65%), M (93%), F (83%), HN (77%), and L (91%). The host range difference is exemplified by the highly permissive growth of HPIV3 in rhesus monkeys, compared to the restricted replication of two different strains of BPIV3 in the same animal (van Wyke Coelingh et al., 1988, *supra*). Although the basis of the host range differences between HPIV3 and BPIV3 remains to be determined, it is likely that they will involve more than one gene and multiple amino acid differences. The involvement of multiple genes and possibly cis-acting regulatory sequences, each involving multiple amino acid or nucleotide differences, gives a very broad basis for

attenuation, one which cannot readily be altered by reversion. This is in contrast to the situation with other live attenuated HPIV3 viruses which are attenuated by one or several point mutations. In this case, reversion of any individual mutation may yield a significant reacquisition of virulence or, in a case where only a single residue specified attenuation, complete reacquisition of virulence.

In exemplary embodiments of the invention described hereinbelow, the background genome or antigenome is an HPIV3 genome or antigenome, and the heterologous gene or genome segment is a N ORF derived from, alternatively, a Ka or SF strain of BPIV3 (which are 99% related in amino acid sequence). The N ORF of the HPIV3 background antigenome is substituted by the counterpart BPIV3 N ORF yielding a novel recombinant human-bovine chimeric PIV cDNA clone. Replacement of the HPIV3 N ORF of HPIV3 with that of BPIV3 Ka or SF results in a protein with approximately 70 amino acid differences (depending on the strain involved) from that of HPIV3 N. N is one of the more conserved proteins, and substitution of other proteins such as P, singly or in combination, would result in many more amino acid differences. The involvement of multiple genes and genome segments each conferring multiple amino acid or nucleotide differences provides a broad basis for attenuation which is highly stable to reversion.

This mode of attenuation contrasts sharply to current HPIV vaccine candidates that are attenuated by one or more point mutations, where reversion of an individual mutation may yield a significant or complete reacquisition of virulence. In addition, several known attenuating point mutations in HPIV typically yield a temperature sensitive phenotype. One problem with attenuation associated with temperature sensitivity is that the virus can be overly restricted for replication in the lower respiratory tract while being under attenuated in the upper respiratory tract. This is because there is a temperature gradient within the respiratory tract, with temperature being higher (and more restrictive) in the lower respiratory tract and lower (less restrictive) in the upper respiratory tract. The ability of an attenuated virus to replicate in the upper respiratory tract can result in complications including congestion, rhinitis, fever and otitis media, whereas overattenuation in the lower respiratory tract can reduce immunogenicity. Thus, attenuation achieved solely by temperature sensitive mutations may not be ideal. In contrast, host range mutations present in human-bovine chimeric PIV of the invention will not in most cases confer temperature sensitivity. Therefore, the novel method of PIV attenuation provided by the invention will be more stable genetically and phenotypically

and less likely to be associated with residual virulence in the upper respiratory tract compared to other known PIV vaccine candidates.

Surprisingly, both the Ka and SF HPIV3/BPIV3 chimeric recombinants involving the N ORF replacement were viable. Since the N protein of Ka or SF strain BPIV3 differs in 70 of 515 amino acid residues, respectively, from that of the JS strain of HPIV3. It was therefore unexpected that a bovine N protein with this level of amino acid sequence divergence could efficiently interact with the HPIV3 RNA, or with other HPIV3 proteins that constitute the functional replicase/transcriptase. Equally surprising was the finding that the Ka and SF chimeric viruses replicated as efficiently in cell culture as either HPIV3 or BPIV3 parent indicating that the chimeric recombinants did not exhibit gene incompatibilities that restricted replication *in vitro*. This property of efficient replication *in vitro* is important since it permits efficient manufacture of this biological.

Also surprising is the observation, based on the studies hereinbelow, that the Ka and the SF HPIV3/BPIV3 chimeric recombinants (termed cKa and cSF), bearing only one bovine gene, are nearly equivalent to their BPIV3 parents in the degree of host range restriction in the respiratory tract of the rhesus monkey. In particular, the cKa and cSF viruses exhibited approximately a 60-fold or 30-fold reduction, respectively, in replication in the upper respiratory tract of rhesus monkeys compared to replication of HPIV3. Based on this finding, it is possible that other BPIV3 genes will also confer desired levels of host range restriction within human-bovine chimeric PIV of the invention. Thus, according to the methods herein, a list of attenuating determinants will be readily identified in heterologous genes and genome segments of both HPIV and BPIV that will confer, in appropriate combination, an optimal level of host range restriction and immunogenicity on human-bovine chimeric PIV selected for vaccine use. In preferred vaccine recombinants, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model for PIV replication in humans, e.g., hamsters or rhesus monkeys, may be reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-100-fold and up to 1,000-fold or greater overall (e.g., as measured between 3-8 days following infection) compared to growth of the corresponding wild-type or mutant parental PIV strain.

Confirming the unexpected nature and advantages provided by the human-bovine chimeric PIV of the invention, both the cKa and cSF induced a high level of protection against HPIV3 challenge in the respiratory tract of rhesus monkeys, despite the exceptional degree of restriction of replication exhibited by these viruses in this model for

human PIV infection and protection. In particular, previous infection with either chimeric virus induced a high level of resistance to replication of the rJS challenge virus in both the upper and lower respiratory tract. Infection of monkeys with cKa elicited a high degree of protection as indicated by an approximate 300-fold reduction of replication of wild type HPIV3 (rJS) in the upper respiratory tract, and an approximate 1000-fold reduction in the lower tract compared to uninoculated control monkeys. Monkeys infected with cSF manifested a 2000-fold reduction of replication of rJS in the upper respiratory tract, and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys. The levels of protection elicited by cKa or cSF were comparable to those seen in monkeys previously infected with either the bovine or the human PIV parent. Thus, infection with human-bovine chimeric PIV of the invention provides a high level of protection in the upper and lower respiratory tract of monkeys, and both chimeric viruses represent promising vaccine candidates. In other preferred vaccine recombinants, the immunogenic activity of human-bovine chimeric PIV will be balanced against the level of attenuation to achieve useful vaccine candidates, and will typically be marked by a reduction of replication of challenge virus, e.g., rJS in the lower and/or upper respiratory tract by about 50-100-fold, 100-500-fold, preferably about 500-2,000-fold and up to 3,000-fold or greater overall (e.g., as measured between 3-8 days post-challenge). Thus, the recombinant vaccine viruses of the invention maintain immunogenicity while exhibiting concomitant reductions in replication and growth. This surprising assemblage of phenotypic traits is highly desired for vaccine development.

The observation that the N gene from two independent strains of BPIV3 confers an attenuation phenotype on HPIV3 for the rhesus monkey indicates that this is likely a property shared by N genes of other BPIV strains. Accordingly, within the methods of the invention any BPIV gene or genome segment, singly or in combination with one or more other BPIV gene(s) or genome segment(s), can be combined with HPIV sequences to produce an attenuated HPIV3/BPIV3 chimeric recombinant virus suitable for use as a vaccine virus. In preferred embodiments, all HPIVs, including HPIV1, HPIV2, HPIV3 and variant strains thereof, are useful recipients for attenuating BPIV gene(s) and/or genome segment(s). In general, the HPIV genes selected for inclusion in a HPIV3/BPIV3 chimeric virus will include one or more of the protective antigens, such as the HN or F glycoproteins.

Alternative human-bovine chimeric PIV of the invention will contain protective antigenic determinants of HPIV1 or HPIV2. This may be achieved, for

example, by expression of an HN and/or F gene of HPIV1 or HPIV2 as an extra gene(s) in an attenuated HPIV3/BPIV3 chimeric recombinant. Alternatively, it is possible to use a HPIV3/HPIV1 or a HPIV3/HPIV2 antigenic chimeric virus, in which the HPIV1 or HPIV2 HN and/or F genes replace their PIV3 counterpart(s) (Skiadopoulos et al., 1999a, *supra*; Tao et al., 1999, *supra*; and U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; each incorporated herein by reference), as a recipient or background virus for one or more heterologous, attenuating bovine gene(s) or genome segment(s), for example a Ka or SF N gene or genome segment. Such antigenic chimeric viruses will be attenuated by the bovine N gene, but will induce immunity to the HPIV1 or HPIV2 virus.

10 In this context, a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45L* (Skiadopoulos et al., 1998, *supra*; Tao et al., 1998, *supra*; Tao et al., 1999, *supra*).

15 rPIV3-1.*cp45L* was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1*cp45*, has also been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations in HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al., 1999a, *supra*).

20 Still other HPIV/BPIV chimeric recombinants will incorporate two or more BPIV genes or genome segments, in any combination, up to and including all of the BPIV genome other than selected genes or antigenic determinants selected from HN or F gene(s) and genome segment(s), which could come from a human HPIV1, HPIV2, or HPIV3 virus. Yet additional embodiments of the invention are directed to human-bovine

25 chimeric PIV incorporating attenuating genes from other animal PIVs, such as murine PIV1, the canine SV5 PIV2 virus, or another avian or mammalian PIV in combination with a HPIV backbone, alternatively including a chimeric HPIV backbone, from HPIV1, HPIV2, and/or HPIV3.

30 In other detailed aspects of the invention, human-bovine chimeric PIV are employed as vectors for protective antigens of heterologous pathogens, including other PIVs and non-PIV viruses and non-viral pathogens. Within these aspects, the bovine-human chimeric genome or antigenome comprises a partial or complete PIV "vector genome or antigenome" combined with one or more heterologous genes or genome segments encoding one or more antigenic determinants of one or more heterologous

pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference). The heterologous pathogen in this context may be a heterologous PIV and the heterologous gene(s) or genome segment(s) can be selected to encode one or more PIV N, P, C, D, V, M, F, SH (where applicable), HN and/or L protein(s), as well as protein domains, fragments, and immunogenic regions or epitopes. PIV vector vaccines thus constructed may elicit a polyspecific immune response and may be administered simultaneously or in a coordinate administration protocol with other vaccine agents.

In exemplary embodiments of the invention, human-bovine chimeric PIV may comprise a vector genome or antigenome that is a partial or complete HPIV genome or antigenome, which is combined with or is modified to incorporate one or more heterologous genes or genome segments encoding antigenic determinant(s) of one or more heterologous PIV(s), including heterologous HPIVs selected from HPIV1, HPIV2, or HPIV3. In more detailed aspects, the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more heterologous HPIV(s). Typically, the chimeric genome or antigenome incorporates one or more gene(s) or genome segment(s) of a BPIV that specifies attenuation.

In exemplary aspects of the invention, the bovine-human chimeric PIV incorporates one or more HPIV1 or HPIV2 genes or genome segments that encode(s) one or more HN and/or F glycoproteins or antigenic domains, fragments or epitopes thereof within a partial or complete HPIV3 vector genome or antigenome. In more detailed aspects, both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome. Such recombinant constructs can be used to produce vaccine virus directly, or can be further modified by addition or incorporation of one or more genes or gene segments encoding one or more antigenic determinants. Such constructs for the production of vaccine viruses typically incorporate one or more heterologous gene(s) or genome segment(s) of a BPIV that specifies attenuation, for example an open reading frame (ORF) encoding an attenuating BPIV protein, such as N. Certain human-bovine chimeric PIV of the invention may be employed as vectors for generating specific vaccines to HPIV2, for example wherein a transcription unit comprising an open reading frame (ORF) of an HPIV2 HN gene is added to or incorporated within a chimeric HPIV3-

1 vector genome or antigenome and the chimeric construct is attenuated by incorporation of a BPIV gene or genome segment.

Within related aspects of the invention, the vector genome or antigenome is a partial or complete BPIV genome or antigenome, and the heterologous genes or genome segments encoding the antigenic determinant(s) is/are of one or more HPIV(s). Typically, the determinant(s) is/are selected from HPIV1, HPIV2 or HPIV3 HN and F glycoproteins, but antigenic domains, fragments and epitopes of these and other antigenic proteins are also useful. In certain embodiments, one or more genes or genome segments encoding one or more antigenic determinant(s) of HPIV2 is/are added to or substituted within the partial or complete BPIV vector genome or antigenome. Alternatively, a plurality of heterologous genes or genome segments encoding antigenic determinants of multiple HPIVs may be added to or incorporated within the partial or complete BPIV vector genome or antigenome.

In yet additional aspects of the invention, human-bovine chimeric PIV are provided as vectors for a range of non-PIV pathogens (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference). The vector genome or antigenome for use within these aspects of the invention may comprise a partial or complete BPIV or HPIV genome or antigenome, and the heterologous pathogen may be selected from measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses.

For example, a HPIV or BPIV vector genome or antigenome for constructing bovine-human chimeric PIV of the invention may incorporate heterologous antigenic determinant(s) selected from the measles virus HA and F proteins, or antigenic domains, fragments and epitopes thereof. In exemplary embodiments, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to or incorporated within a BPIV or HPIV3 vector genome or antigenome.

Alternatively bovine-human chimeric PIV of the invention may be used as vectors to incorporate heterologous antigenic determinant(s) from respiratory syncytial virus (RSV), for example by incorporating one or more genes or genome segments that encode(s) RSV F and/or G glycoprotein or immunogenic domain(s) or epitope(s) thereof. In this context, the cloning of RSV cDNA and other disclosure is provided in U.S.

Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application Serial No. 09/291,894, filed on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999; Collins, et al., 1995, *supra*; Bukreyev, et al., J. Virol. 70:6634-6641, 1996; Juhasz et al., 1997, *supra*; Durbin et al., 1997a, *supra*; He et al., 1997, *supra*; Baron et al., 1997, *supra*; Whitehead et al., 1998a, *supra*; Whitehead et al., 1998b, *supra*; Jin et al., 1998, *supra*; and Whitehead et al., 1999, *supra*; and Bukreyev et al., Proc. Natl. Acad. Sci. USA 96:2367-2372, 1999, each incorporated herein by reference in its entirety for all purposes).

According to this aspect of the invention, human-bovine chimeric PIV are provided which incorporate at least one antigenic determinant from a heterologous PIV or non-PIV pathogen. For example, one or more individual gene(s) or genome segment(s) of HPIV3 may be replaced with counterpart gene(s) or genome segment(s) from human RSV, or an RSV gene or genome segment can be inserted or added as an supernumerary gene. Alternatively, a selected, heterologous genome segment, e.g. encoding a cytoplasmic tail, transmembrane domain or ectodomain of an RSV glycoprotein, is substituted for a counterpart genome segment in, e.g., the same gene in HPIV3 or within a different gene in HPIV3, or added within a non-coding sequence of the HPIV3 genome or antigenome to yield a chimeric PIV-RSV glycoprotein. In one embodiment, a genome segment from an F gene of human RSV is substituted for a counterpart HPIV3 genome segment to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of PIV fused to an ectodomain of RSV to yield a novel attenuated virus, and/or a multivalent vaccine immunogenic against both PIV and RSV.

As noted above, it is often desirable to adjust the attenuation phenotype in human-bovine chimeric PIV of the invention by introducing additional mutations that increase or decrease attenuation or otherwise alter the phenotype of the chimeric virus. Detailed descriptions of the materials and methods for producing recombinant PIV from cDNA, and for making and testing the full range of mutations and nucleotide modifications set forth herein as supplemental aspects of the present invention, provided

in, e.g., Durbin et al., 1997a, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997; each incorporated herein by
5 reference. In particular, these documents describe methods and procedures for mutagenizing, isolating and characterizing PIV to obtain attenuated mutant strains (e.g., temperature sensitive (ts), cold passaged (cp) cold-adapted (ca), small plaque (sp) and host-range restricted (hr) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype. In conjunction with these methods, the foregoing
10 documents detail procedures for determining replication, immunogenicity, genetic stability and protective efficacy of biologically derived and recombinantly produced attenuated human PIV in accepted model systems, including murine and non-human primate model systems. In addition, these documents describe general methods for developing and testing immunogenic compositions, including monovalent and bivalent
15 vaccines, for prophylaxis and treatment of PIV infection. Methods for producing infectious recombinant PIV by construction and expression of cDNA encoding a PIV genome or antigenome coexpressed with essential PIV proteins are also described in the above-incorporated documents, which include description of the following exemplary plasmids that may be employed to produce infectious PIV viral clones: p3/7(131) (ATCC
20 97990); p3/7(131)2G (ATCC 97889); and p218(131) (ATCC 97991); each deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and granted the above identified accession numbers.

Also disclosed in the above-incorporated references are methods for
25 constructing and evaluating infectious recombinant PIV that are modified to incorporate phenotype-specific mutations identified in biologically-derived PIV mutants, e.g., cold passaged (cp), cold adapted (ca), host range restricted (hr), small plaque (sp), and/or temperature sensitive (ts) mutants, for example the JS HPIV3 cp 45 mutant strain. Other mutations may be attenuating without an auxiliary marker phenotype. Mutations
30 identified in these mutants can be readily adopted in human-bovine chimeric PIV. In exemplary embodiments, one or more attenuating mutations occur in the polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS cp45. Preferably, these mutations are incorporated in human-bovine chimeric PIV of the invention by an identical, or conservative, amino acid substitution as identified in the

biological mutant. Thus, PIV recombinants may incorporate a mutation wherein Tyr₉₄₂ is replaced by His, Leu₉₉₂ is replaced by Phe, and/or Thr₁₅₅₈ is replaced by Ile.

Substitutions that are conservative to these replacement amino acids are also useful to achieve a desired mutant phenotype

5 Other exemplary mutations adopted from a biologically derived PIV mutant include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS cp45. In more detailed aspects, these mutations are represented as Val₉₆ to Ala or Ser₃₈₉ to Ala or substitutions that are conservative thereto. Also useful within recombinant PIV of the invention are amino acid
10 substitution in the C protein, e.g., a mutation at a position corresponding to Ile₉₆ of JS cp45, preferably represented by an identical or conservative substitution of Ile₉₆ to Thr. Further exemplary mutations adopted from biologically derived PIV mutants include one mutation in the M gene such as Pro₁₉₉ in JS cp45, or more mutations in the F protein, including mutations adopted from JS cp45 at a position corresponding to residues Ile₄₂₀
15 or Ala₄₅₀ of JS cp45, preferably represented by acid substitutions Ile₄₂₀ to Val or Ala₄₅₀ to Thr or substitutions conservative thereto. Other human-bovine chimeric PIV within the invention adopt one or more amino acid substitutions in the HN protein, as exemplified hereinbelow by a recombinant PIV adopting a mutation at a position corresponding to residue Val₃₈₄ of JS, preferably represented by the substitution Val₃₈₄ to Ala.

20 Yet additional examples within this aspect of the invention include human-bovine chimeric PIV which incorporate one or more mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS cp45. Yet
25 additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS cp45. In more detailed aspects human-bovine chimeric PIV incorporate a T to C change at nucleotide 23, a C to T change at nucleotide 24, a G to T change at nucleotide 28, and/or a T to A change at nucleotide 45.
30 Additional mutations in extragenic sequences are exemplified by a A to T change in N gene start sequence at a position corresponding to nucleotide 62 of JS.

These foregoing exemplary mutations which can be engineered in a human-bovine chimeric PIV of the invention have been successfully engineered and recovered in recombinant PIV, as represented by the recombinant PIV clones designated

rcp45, rcp45 L, rcp45 F, rcp45 M, rcp45 HN, rcp45 C, rcp45 F, rcp45 3'N, 3'NL, and
rcp45 3'NCMFHN (Durbin et al., 1997a, *supra*; Skiadopolos et al., 1998, *supra*;
Skiadopolos et al., *J. Virol.* 73:1374-1381, 1999b; U.S. Patent Application Serial No.
09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed
5 May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S.
Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated
herein by reference). In addition, the above-incorporated references describe construction
of chimeric PIV recombinants, e.g., having the HN and F genes of HPIV1 substituted into
a partial HPIV3 background genome or antigenome, which is further modified to bear one
10 or more of the attenuating mutations identified in HPIV3 JS cp45. One such chimeric
recombinant incorporates all of the attenuating mutations identified in the L gene of cp45.
It has since been shown that all of the cp45 mutations outside of the heterologous
(HPIV1) HN and F genes can be incorporated in a HPIV3-l recombinant to yield an
attenuated, chimeric vaccine candidate.

15 From JS cp45 and other biologically derived PIV mutants, a large "menu"
of attenuating mutations is provided, each of which can be combined with any other
mutation(s) for adjusting the level of attenuation, immunogenicity and genetic stability in
a recombinant PIV bearing C, D, and/or V deletion or knock out mutation(s). In this
context, many recombinant PIVs of the invention will include one or more, and
20 preferably two or more, mutations from biologically derived PIV mutants, e.g., any one or
combination of mutations identified in JS cp45. Preferred PIV recombinants within the
invention will incorporate a plurality and up to a full complement of the mutations present
in JS cp45 or other biologically derived mutant PIV strains. Preferably, these mutations
are stabilized against reversion in human-bovine chimeric PIV by multiple nucleotide
25 substitutions in a codon specifying each mutation.

Additional mutations that may be incorporated in human-bovine chimeric
PIV of the invention are mutations, e.g., attenuating mutations, identified in heterologous
PIV or more distantly related nonsegmented negative stranded RNA viruses. In
particular, attenuating and other desired mutations identified in one negative stranded
30 RNA virus may be "transferred", e.g., introduced by mutagenesis in a corresponding
position within the genome or antigenome of the human-bovine chimeric PIV. Briefly,
desired mutations in one heterologous negative stranded RNA virus are transferred to the
PIV recipient (e.g., bovine or human PIV, respectively). This involves mapping the
mutation in the heterologous virus, thus identifying by sequence alignment the

corresponding site in the recipient RSV, and mutating the native sequence in the PIV recipient to the mutant genotype (either by an identical or conservative mutation), as described in PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference). As this disclosure teaches, it is preferable to modify the recipient genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a similar substitution should be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will involve an identical or conservative amino acid to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the function of the wild-type residue).

Negative stranded RNA viruses from which exemplary mutations are identified and transferred into human-bovine chimeric PIV of the invention include other PIVs (e.g., HPIV1, HPIV2, HPIV3, HPIV4A, HPIV4B and BPIV3), RSV, Sendai virus (SeV), Newcastle disease virus (NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV), rabies virus (RaV) and vesicular stomatitis virus (VSV), among others.

A variety of exemplary mutations for use within the invention are disclosed in the above-incorporated reference, including but not limited to an amino acid substitution of phenylalanine at position 521 of the RSV L protein corresponding to and therefore transferable to a substitution of phenylalanine (or a conservatively related amino acid) at position 456 of the HPIV3 L protein. In the case of mutations marked by deletions or insertions, these can be introduced as corresponding deletions or insertions into the recombinant virus, either within the background genome or antigenome or within the heterologous gene or genome segment incorporated therein. However the particular size and amino acid sequence of the deleted or inserted protein fragment can vary.

Yet additional human-bovine PIV vaccine candidates within the invention can be achieved by modifying the chimeric PIV genome or antigenome to encode an analogous mutation to an attenuating mutation identified in Sendai virus (SeV). In one example, the attenuating mutation comprises an amino acid substitution of phenylalanine

at position 170 of the C protein of SeV. The PIV genome or antigenome is modified to encode an alteration of a conserved residue that corresponds conservatively to the alteration marking the attenuating mutation in the heterologous, SeV mutant. In one embodiment, the mutation is incorporated within a recombinant HPIV3 protein and
5 comprises an amino acid substitution of phenylalanine at position 164 of the C protein of HPIV3.

Various target proteins are amenable to introduction of attenuating mutations from one negative stranded RNA virus at a corresponding site within chimeric human-bovine PIV of the invention. Throughout the order Mononegavirales, five target
10 proteins are strictly conserved and show moderate to high degrees of sequence identity for specific regions or domains. In particular, all known members of the order share a homologous constellation of five proteins: a nucleocapsid protein (N), a nucleocapsid phosphoprotein (P), a nonglycosylated matrix (M) protein, at least one surface glycoprotein (HN, F, H, or G) and a large polymerase (L) protein. These proteins all
15 represent useful targets for incorporating attenuating mutations by altering one or more conserved residues in a protein of the recombinant virus at a site corresponding to the site of an attenuating mutation identified in the heterologous, mutant virus.

In this context, the methods for transferring heterologous mutations into chimeric human-bovine PIV of the invention are based on identification of an attenuating
20 mutation in a first negative stranded RNA virus. The mutation, identified in terms of mutant versus wild-type sequence at the subject amino acid position(s) marking the site of the mutation, provides an index for sequence comparison against a homologous protein in the chimeric virus (either in the background genome or antigenome or in the heterologous gene or gene segment added or substituted therein) that is the target for recombinant
25 attenuation. The attenuating mutation may be previously known or may be identified by mutagenic and reverse genetics techniques applied to generate and characterize biologically-derived mutant virus. Alternatively, attenuating mutations of interest may be generated and characterized *de novo*, e.g., by site directed mutagenesis and conventional screening methods.

30 Each attenuating mutation identified in a negative stranded RNA virus provides an index for sequence comparison against a homologous protein in one or more heterologous negative stranded virus(es). In this context, existing sequence alignments may be analyzed, or conventional sequence alignment methods may be employed to yield sequence comparisons for analysis, to identify corresponding protein regions and amino

acid positions between the protein bearing the attenuating mutation and a homologous protein of a different virus that is the target recombinant virus for attenuation. Where one or more residues marking the attenuating mutation have been altered from a "wild-type" identity that is conserved at the corresponding amino acid position(s) in the target human-bovine chimeric virus protein, the genome or antigenome of the target virus is recombinantly modified to encode an amino acid deletion, substitution, or insertion to alter the conserved residue(s) in the target virus protein and thereby confer an analogous, attenuated phenotype on the recombinant virus.

Within this rational design method for constructing attenuated recombinant negative stranded viruses, the wild-type identity of residue(s) at amino acid positions marking an attenuating mutation in one negative stranded RNA virus may be conserved strictly, or by conservative substitution, at the corresponding amino acid position(s) in the target, human-bovine chimeric virus protein. Thus, the corresponding residue(s) in the target virus protein may be identical, or may be conservatively related in terms of amino acid side-group structure and function, to the wild-type residue(s) found to be altered by the attenuating mutation in the heterologous, mutant virus. In either case, analogous attenuation in the recombinant virus may be achieved according to the methods of the invention by modifying the recombinant genome or antigenome of the target virus to encode the amino acid deletion, substitution, or insertion to alter the conserved residue(s).

In this context, it is preferable to modify the genome or antigenome to encode an alteration of the conserved residue(s) that corresponds conservatively to the alteration marking the attenuating mutation in the heterologous, mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a substitution should be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will be identical or conservative to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the identity and function of the wild-type residue). In the case of mutations marked by deletions or insertions, these can transferred as corresponding deletions or insertions into the recombinant virus, however the particular size and amino acid sequence of the deleted or inserted protein fragment can vary.

Within alternative aspects of the invention, mutations thus transferred from heterologous mutant negative stranded viruses may confer a variety of phenotypes within human-bovine chimeric PIV of the invention, in addition to or associated with the desired, an attenuated phenotype. Thus, exemplary mutations incorporated within

5 recombinant proteins of the virus may confer temperature sensitive (*ts*), cold-adapted (*ca*), small plaque (*sp*), or host range restricted (*hr*) phenotypes, or a change in growth or immunogenicity, in addition to or associated with the attenuated phenotype.

Attenuating mutations in biologically derived PIV and other nonsegmented negative stranded RNA viruses for incorporation within human-bovine chimeric PIV may

10 occur naturally or may be introduced into wild-type PIV strains by well known mutagenesis procedures. For example, incompletely attenuated parental PIV strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to introduce growth restriction mutations, or

15 by selection of a mutagenized virus that produces small plaques (*sp*) in cell culture, as described in the above incorporated references.

By "biologically derived PIV" is meant any PIV not produced by recombinant means. Thus, biologically derived PIV include all naturally occurring PIV, including, e.g., naturally occurring PIV having a wild-type genomic sequence and PIV

20 having allelic or mutant genomic variations from a reference wild-type RSV sequence, e.g., PIV having a mutation specifying an attenuated phenotype. Likewise, biologically derived PIV include PIV mutants derived from a parental PIV by, *inter alia*, artificial mutagenesis and selection procedures.

As noted above, production of a sufficiently attenuated biologically

25 derived PIV mutant can be accomplished by several known methods. One such procedure involves subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a cp mutant or other partially attenuated PIV strain is adapted to efficient growth at a lower temperature

30 by passage in culture. This selection of mutant PIV during cold-passages substantially reduces any residual virulence in the derivative strains as compared to the partially attenuated parent.

Alternatively, specific mutations can be introduced into biologically derived PIV by subjecting a partially attenuated parent virus to chemical mutagenesis,

e.g., to introduce ts mutations or, in the case of viruses which are already ts, additional ts mutations sufficient to confer increased attenuation and/or stability of the ts phenotype of the attenuated derivative. Means for the introduction of ts mutations into PIV include replication of the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical mutagens can also be used. Attenuation can result from a ts mutation in almost any PIV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene.

The level of temperature sensitivity of replication in exemplary attenuated PIV for use within the invention is determined by comparing its replication at a permissive temperature with that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the shutoff temperature. In experimental animals and humans, both the replication and virulence of PIV correlate with the mutant's shutoff temperature.

The JS cp45 HPIV3 mutant has been found to be relatively stable genetically, highly immunogenic, and satisfactorily attenuated. Nucleotide sequence analysis of this biologically derived and recombinant viruses incorporating various individual and combined mutations found therein, indicates that each level of increased attenuation is associated with specific nucleotide and amino acid substitutions. The above-incorporated references also disclose how to routinely distinguish between silent incidental mutations and those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious PIV clones. This process coupled with evaluation of phenotype characteristics of parental and derivative virus identifies mutations responsible for such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc.

Mutations thus identified are compiled into a "menu" and are then introduced as desired, singly or in combination, to adjust a human-bovine chimeric PIV to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc., as desired. In accordance with the foregoing description, the ability to produce infectious PIV from cDNA permits introduction of specific engineered changes within human-bovine chimeric PIV. In particular, infectious, recombinant PIVs are employed for identification of specific mutation(s) in biologically derived, attenuated PIV strains, for example mutations which specify ts, ca, att and other

phenotypes. Desired mutations are thus identified and introduced into recombinant, human-bovine chimeric PIV vaccine strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, whereafter the phenotypes of rescued recombinant viruses containing the introduced mutations can be readily determined.

By identifying and incorporating specific, biologically derived mutations associated with desired phenotypes, e.g., a cp or ts phenotype, into infectious PIV clones, the invention provides for other, site-specific modifications at, or within close proximity to, the identified mutation. Whereas most attenuating mutations produced in biologically derived PIV are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into biologically derived or recombinant PIV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from 1 to 3, up to about 5-15 or more altered nucleotides (e.g., altered from a wild-type PIV sequence, from a sequence of a selected mutant PIV strain, or from a parent recombinant PIV clone subjected to mutagenesis). Such site-specific mutations may be incorporated at, or within the region of, a selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within a PIV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc. Site-specific PIV mutants typically retain a desired attenuating phenotype, but may additionally exhibit altered phenotypic characteristics unrelated to attenuation, e.g., enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant PIV designed to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant PIV clone, yielding a biologically derived or recombinant PIV having genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream or downstream, e. g., from 1 to 3, 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, e.g., to construct or ablate an existing cis-acting regulatory element.

an initiation codon, or modifying an editing site. These forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, these methods provide yet additional, novel types of attenuating mutations which ablate expression of a viral gene that is not one of the major viral protective antigens. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Several other gene knock-outs for the C, D, and/or V ORF(s) deletion and knock out mutants can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer et al., *Virology* 216:309-316, 1996; Radecke et al., *Virology* 217:418-421, 1996; and Kato et al., 1987a, *supra*; and Schneider et al., 1997, *supra*; each incorporated herein by reference).

These and other nucleotide modifications in human-bovine chimeric PIV may alter small numbers of bases (e.g., from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (e.g., 50-100, 100-300, 300-500, 500-1,000 bases), or nearly complete or complete genes (e.g., 1,000-1,500 nucleotides, 1,500-2,500 nucleotides, 2,500-5,000, nucleotides, 5,000-6,500 nucleotides or more) in the donor or recipient genome or antigenome, depending upon the nature of the change (i.e., a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large block(s) of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In related aspects, the invention provides for supplementation of mutations adopted into a recombinant PIV clone from biologically derived PIV, e.g., cp and ts mutations, with additional types of mutations involving the same or different genes in a further modified PIV clone. Each of the PIV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a human-bovine chimeric PIV exhibiting novel vaccine characteristics. Thus, in addition to or in combination with attenuating mutations adopted from biologically derived PIV mutants, the present invention also provides a range of additional methods for attenuating or otherwise modifying the phenotype of human-bovine chimeric PIV based on recombinant engineering of infectious PIV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a chimeric PIV genome or antigenome

for incorporation into infectious clones. More specifically, to achieve desired structural and phenotypic changes in recombinant PIV, the invention allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or plurality of nucleotides from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole gene(s) or genome segment(s), within a human-bovine chimeric PIV clone.

Thus provided are modifications in the human-bovine chimeric PIV which simply alter or ablate expression of a selected gene, e.g., by introducing a termination codon within a selected PIV coding sequence or altering its translational start site or RNA editing site, changing the position of a PIV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (e.g., by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (e.g., growth, temperature restrictions on transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected gene(s), or translation of selected RNA(s). In this context, any PIV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant PIV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. As for coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, e.g., by the use of minireplicons and recombinant PIV.

In addition, a variety of other genetic alterations can be produced in a PIV genome or antigenome for incorporation into human-bovine chimeric PIV, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV, e.g., to adjust growth, attenuation, immunogenicity, genetic stability or provide other advantageous structural and/or phenotypic effects. These additional types of mutations are also disclosed in the foregoing incorporated references and can be readily engineered into human-bovine chimeric PIV of the invention.

In addition to these changes, the order of genes in a human-bovine chimeric PIV can be changed, a PIV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic

regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into human-bovine chimeric PIV of the invention include mutations directed toward cis-acting signals, which can be identified, e.g., by mutational analysis of PIV minigenomes. For example, insertional and deletional analysis of the leader and trailer and flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also has identified many mutations which affect RNA replication or transcription. Any of these mutations can be inserted into a human-bovine chimeric PIV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of PIV minigenomes as described in the above-incorporated references.

Additional mutations within the human-bovine chimeric PIV involve replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific PIV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Hans et al., Current Biol. 6:315-324, 1996, incorporated herein by reference). Optimization by recombinant methods of the codon usage of the mRNAs encoding the HN and F proteins of PIV, which are the major protective antigens, will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected PIV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate PIV gene expression by specifying up- or down-regulation of translation (Kozak et al., J. Mol. Biol. 196:947-950, 1987). Alternatively, or in combination with other PIV modifications disclosed herein, gene expression of a human-bovine chimeric PIV can be modulated by altering a transcriptional GS or GE signal of any selected gene(s) of the virus. In alternative embodiments, levels of gene expression in the human-bovine chimeric PIV are modified at the level of transcription. In one aspect, the position

of a selected gene in the PIV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more
5 typically four-fold, up to ten-fold or more compared to wild-type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel human-bovine chimeric PIV having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as
10 increased antigen expression.

Infectious human-bovine chimeric PIV clones of the invention can also be engineered according to the methods and compositions disclosed herein to enhance immunogenicity and induce a level of protection greater than that provided by infection with a wild-type PIV or a parent PIV. For example, an immunogenic epitope from a
15 heterologous PIV strain or type, or from a non-PIV source such as RSV, can be added to a recombinant clone by appropriate nucleotide changes in the polynucleotide sequence encoding the genome or antigenome. Alternatively, mutant PIV of the invention can be engineered to add or ablate (e.g., by amino acid insertion, substitution or deletion) immunogenic proteins, protein domains, or forms of specific proteins associated with
20 desirable or undesirable immunological reactions.

Within the methods of the invention, additional genes or genome segments may be inserted into or proximate to the human-bovine chimeric PIV genome or antigenome. These genes may be under common control with recipient genes, or may be under the control of an independent set of transcription signals. Genes of interest include
25 the PIV genes identified above, as well as non-PIV genes. Non-PIV genes of interest include those encoding cytokines (e.g., IL-2 through IL-18, especially IL-2, IL-6 and IL-12, IL-18, etc.). gamma-interferon, and proteins rich in T helper cell epitopes. These additional proteins can be expressed either as a separate protein, or as a supernumerary copy of an existing PIV proteins, such as HN or F. This provides the ability to modify
30 and improve the immune responses against PIV both quantitatively and qualitatively.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within a human-bovine chimeric PIV yield highly stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of

resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (i.e., not essential for in vitro growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., 1997a, *supra*). Ablation of such genes in vaccine viruses is expected to
5 reduce virulence and pathogenesis and/or improve immunogenicity.

In another aspect of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating human-bovine chimeric PIV-encoding cDNA) are provided for producing an isolated infectious PIV. Using these compositions and methods, infectious PIV are generated from a PIV genome or antigenome, a nucleocapsid
10 (N) protein, a nucleocapsid phosphoprotein (P), and a large (L) polymerase protein. In related aspects of the invention, compositions and methods are provided for introducing the aforementioned structural and phenotypic changes into a recombinant PIV to yield infectious, attenuated vaccine viruses.

Introduction of the foregoing defined mutations into an infectious, human-
15 bovine chimeric PIV clone can be achieved by a variety of well known methods. By “infectious clone” with regard to DNA is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of serving as template to produce the genome of an infectious virus or subviral particle. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed
20 mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA subfragments to assemble a complete antigenome or genome cDNA as described herein has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or any
25 subfragment thereof can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, CA) or a method using a double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, CA), or by the polymerase chain reaction employing either an
30 oligonucleotide primer or template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and available for use in producing the mutations of interest in the PIV antigenome or genome cDNA. Mutations can vary from

single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Thus, in one illustrative embodiment mutations are introduced by using the Muta-gene phagemid *in vitro* mutagenesis kit available from Bio-Rad. In brief, cDNA encoding a portion of a PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome fragment is then amplified and the mutated piece is then reintroduced into the full-length genome or antigenome clone.

Infectious PIV of the invention are produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode a PIV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins necessary to generate a transcribing, replicating nucleocapsid. Among the viral proteins useful for coexpression to yield infectious PIV are the major nucleocapsid protein (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein, fusion protein (F), hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M) protein. Also useful in this context are products of the C, D and V ORFs of PIV.

cDNAs encoding a PIV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid.

In some embodiments of the invention the genome or antigenome of a recombinant PIV (rPIV) need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the PIV genome or antigenome encodes all functions necessary for

viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

By "recombinant PIV" is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced therefrom. The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV genome or antigenome, the genome or antigenome is coexpressed with those PIV N, P and L proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Synthesis of PIV antigenome or genome together with the above-mentioned viral proteins can also be achieved *in vitro* (cell-free), e.g., using a combined transcription-translation reaction, followed by transfection into cells. Alternatively, antigenome or genome RNA can be synthesized *in vitro* and transfected into cells expressing PIV proteins.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating PIV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded by the PIV cDNA. For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the PIV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the PIV cDNA to provide antigenic diversity from the helper virus, such as in the HN or F glycoprotein genes.

In alternate embodiments of the invention, the N, P, L and other desired PIV proteins are encoded by one or more non-viral expression vectors, which can be the same or separate from that which encodes the genome or antigenome. Additional proteins may be included as desired, each encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a promoter for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., *Virology* 210:202-205, 1995, incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, San Diego, 1990; each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., *J. Virol.* 69:5677-5686, 1995), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., *Nature* 350:434-436, 1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-PIV nucleotides. The left- and right-hand ends are then joined via a common restriction site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites.

- 5 Alternatively, such techniques as site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome include reverse transcription-PCR using improved PCR conditions (e.g., as described in
10 Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994, incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

15 Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical insertion, transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells
20 by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., New York, 1987), cationic lipid-mediated
25 transfection (Hawley-Nelson et al., Focus 15:73-79, 1993) or a commercially available transfection reagent, e.g., LipofectACE® (Life Technologies, Gaithersburg, MD) or the like (each of the foregoing references are incorporated herein by reference in its entirety).

As noted above, in some embodiments of the invention the N, P, L and other desired PIV proteins are encoded by one or more helper viruses which is
30 phenotypically distinguishable from that which encodes the genome or antigenome. The N, P, L and other desired PIV proteins can also be encoded by one or more expression vectors which can be the same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as

desired, encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome.

By providing infectious clones of PIV the invention permits a wide range of alterations to be recombinantly produced within the PIV genome (or antigenome), yielding defined mutations which specify desired phenotypic changes. By “infectious clone” is meant cDNA or its product, synthetic or otherwise, RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic RNA capable of serving as a template to produce the genome of infectious viral or subviral particles. As noted above, defined mutations can be introduced by a variety of conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA subjects provide for better ease of manipulation than large cDNA subjects, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or a template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a PIV antigenome or genome cDNA of the invention.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid in vitro mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding an PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, e.g., cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, e.g., sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, e.g., epitopic sites, to about 50, 75, 100, 200-500, and 500-1,500 or more nucleotides.

The ability to introduce defined mutations into infectious PIV has many applications, including the manipulation of PIV pathogenic and immunogenic mechanisms. For example, the functions of PIV proteins, including the N, P, M, F, HN, and L proteins and C, D and V ORF products, can be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the invention. The effects of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing PIV minigenomes (Dimock et al., *J. Virol.* 67:2772-2778, 1993, incorporated herein by reference in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.

Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant PIV of the invention (e.g., substitutions of a genome segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or functional relation to an existing, "counterpart" gene or genome segment from the same or different PIV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild-type or parental PIV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of

another PIV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, “counterpart” genes, genome segments, proteins or protein regions, are typically from heterologous sources (e.g., from different PIV genes, or representing the same (i.e., homologous or allelic) gene or genome segment in different PIV types or strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant PIV within the invention, often share substantial sequence identity with a selected polynucleotide “reference sequence,” e.g., with another selected counterpart sequence. As used herein, a “reference sequence” is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a “comparison window” to identify and compare local regions of sequence similarity. A “comparison window”, as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman (Adv. Appl. Math.

2:482, 1981; incorporated herein by reference), by the homology alignment algorithm of Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970; incorporated herein by reference), by the search for similarity method of Pearson & Lipman, (Proc. Natl. Acad. Sci. USA 5:2444, 1988; incorporated herein by reference), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI, incorporated herein by reference), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions encoded by recombinant PIV of the invention are also typically selected to have conservative relationships, i.e. to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (i.e., conservative substitutions) at corresponding positions. The term "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent

sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein follow conventional usage (Immunology - A Synthesis, 2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991, incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α , α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

To select candidate vaccine viruses according to the invention, the criteria of viability, attenuation and immunogenicity are determined according to well known methods. Viruses which will be most desired in vaccines of the invention must maintain viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a vaccinee sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus. The recombinant PIV of the invention are not only viable

and more appropriately attenuated than previous vaccine candidates, but are more stable genetically *in vivo*--retaining the ability to stimulate a protective immune response and in some instances to expand the protection afforded by multiple modifications, e.g., induce protection against different viral strains or subgroups, or protection by a different immunologic basis, e.g., secretory versus serum immunoglobulins, cellular immunity, and the like.

Recombinant PIV of the invention can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus (e.g., a multiply attenuated, biologically derived or recombinant PIV) is tested, e.g., level of replication, for temperature sensitivity of virus replication, i.e. its phenotype, and for the small plaque or other desired phenotype. Modified viruses are further tested in animal models of PIV infection. A variety of animal models have been described and are summarized in various references incorporated herein. PIV model systems, including rodents and non-human primates, for evaluating attenuation and immunogenic activity of PIV vaccine candidates are widely accepted in the art, and the data obtained therefrom correlate well with PIV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the invention also provides isolated, infectious recombinant PIV viral compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to PIV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium where it can be propagated and characterized in a controlled setting. For example, attenuated PIV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For vaccine use, recombinant PIV produced according to the present invention can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg⁺⁺ and HEPES, with or without adjuvant, as further described below.

PIV vaccines of the invention contain as an active ingredient an immunogenically effective amount of PIV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPLTM (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV virus proteins, e.g., F and HN glycoproteins. As a result of the vaccination with an immunogenically effective amount of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinating strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more

commonly from about 10^4 to 10^6 PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be
5 combined with viruses of other PIV serotypes or strains to achieve protection against multiple PIV serotypes or strains. Alternatively, protection against multiple PIV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered
10 simultaneously, but they may also be administered separately. Immunization with one strain may protect against different strains of the same or different serotype.

In some instances it may be desirable to combine the PIV vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. In another aspect of the invention the PIV can be employed as a
15 vector for protective antigens of other pathogens, such as respiratory syncytial virus (RSV) or measles virus, by incorporating the sequences encoding those protective antigens into the PIV genome or antigenome which is used to produce infectious PIV, as described herein (see, e.g., U.S. Provisional Patent Application Serial No. 60/170,195, filed December 10, 1999 by Murphy et al., incorporated herein by reference).

In all subjects, the precise amount of recombinant PIV vaccine
20 administered, and the timing and repetition of administration, will be determined based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6
25 PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated PIV sufficient to effectively stimulate or induce an anti-PIV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As
30 with administration to rhesus monkeys, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at levels approximately 10-fold or more lower than wild-type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated PIV.

In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) PIV infection. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be indicated for administration to different recipient groups. For example, an engineered PIV strain expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

PIV vaccines produced in accordance with the present invention can be combined with viruses expressing antigens of another subgroup or strain of PIV to achieve protection against multiple PIV subgroups or strains. Alternatively, the vaccine virus may incorporate protective epitopes of multiple PIV strains or subgroups engineered into one PIV clone, as described herein.

The PIV vaccines of the invention elicit production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild-type PIV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host-engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup.

Preferred PIV vaccine candidates of the invention exhibit a very substantial diminution of virulence when compared to wild-type virus that is circulating naturally in humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence

is sufficiently abrogated such that severe lower respiratory tract infections in the vaccinated or incidental host do not occur.

The level of attenuation of PIV vaccine candidates may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type PIV or other attenuated PIV which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, or rhesus monkey, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods for determining levels of PIV in the nasopharynx of an infected host are well known in the literature.

Levels of induced immunity provided by the vaccines of the invention can also be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on these measurements, vaccine dosages can be adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be advantageous for different recipient groups. For example, an engineered PIV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In yet another aspect of the invention the PIV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant PIV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls PIV expression. The infectious PIV produced by coexpressing the recombinant PIV genome or antigenome with the N, P, L and other desired PIV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant PIV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products which may

be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

The following examples are provided by way of illustration, not limitation.

EXAMPLE I

Construction of cDNAs Encoding a Chimeric HPIV3/BPIV3 Antigenome and Recovery of Infectious Virus

The following three examples document studies to identify which of the proteins of BPIV3 contribute to its host range restriction in primates. To illustrate these methods, the N protein of the wild type HPIV3 virus was replaced with its counterpart from BPIV3. This exchange was accomplished using a reverse genetics system for recovery of infectious PIV from cDNA as described above. The studies were initiated with the N gene of BPIV3 because this protein possesses an intermediate level of amino acid sequence difference from its HPIV3 counterpart compared to other HPIV3 and BPIV3 proteins (see Example I).

A chimeric recombinant virus was constructed in which the N ORF of the JS strain of HPIV3 was replaced by that of either the Ka or SF strain of BPIV3 (Fig. 1). These chimeric viruses possess the HN and F glycoproteins of the HPIV3 parent and will induce a high level of immunity to HPIV3 in primates. Both chimeric viruses were successfully recovered. Both grew to high titer in cell culture and both were found to be attenuated in rhesus monkeys. Thus, the N protein was identified as an exemplary protein that contributes to the host range phenotype of BPIV3. Immunization of rhesus monkeys with either the Ka or SF chimeric recombinant virus induced a high level of resistance to the replication of HPIV3 used as a wild type challenge.

The present invention, therefore, establishes the usefulness of reverse genetics methods to generate chimeric human-bovine PIV virus that combines the host range attenuation properties of BPIV3 and the immunogenicity of the HPIV3 HN and F protective antigens. Immunization of humans with such a chimeric recombinant will redress the problem of suboptimal immunogenicity of the BPIV3 vaccine previously observed in humans.

5 The complete consensus nucleotide sequence for each of the Ka or SF
BPIV3 strains was determined from RT-PCR products generated from virion RNA.
These sequences are set forth in Figures 6A-6G, and Figures 7A-7G, respectively. The
full length cDNA encoding a complete 15456 nucleotide (nt) antigenomic RNA of BPIV3
Ka is set forth in Figures 6A-6G herein (see also GenBank accession #AF178654). The
GenBank sequence for BPIV3 kansas strain differs from the sequence of the exemplary
cDNA in two positions at nucleotide 21 and 23. Both, the published sequence and the
sequence in the exemplary cDNA occur naturally in kansas strain virus population with
similar frequencies. The former cDNA contains a sequence beginning at nucleotide 18,
10 ACTGGTT, whereas the corresponding published sequence (GenBank accession
#AF178654; Figures 6A-6G) reads ACTTGCT (SEQ ID NO. 1) (differing nucleotides at
positions 21 and 23 are underscored).

To construct consensus nucleotide sequences for the Ka and SF BPIV3
strains, virion RNA was subjected to reverse transcription using the Superscript II
15 Preamplification System (Life Technologies, Gaithersburg, MD) and 200 ng of random
hexamer primers. PCR was carried out on the first strand product using the Advantage
cDNA PCR kit (Clontech Laboratories, Palo Alto, CA). Ka and SF genomes were each
amplified by PCR in 3 or 4 overlapping fragments using primers homologous to regions
of RNA conserved among previously-published paramyxovirus sequences. Each primer
20 pair was constructed to include matching restriction enzyme sites (not represented in the
sequence targeted for amplification).

A separate random library was generated for each amplicon by digesting a
set of PCR products with the appropriate restriction enzyme, followed by gel-purification,
ligation of the products into tandem arrays and sonication. A random library was
25 generated from this pool of sheared cDNA sequences by cloning a subset (approx. 500 bp
fragments) into M 13. The nucleotide sequences of cDNA inserts were determined by
automated DNA sequencing using the Taq DYE Deoxy Terminator cycle sequencing kit
(ABI, Foster City, CA). A continuous sequence (contig) was assembled for each of the
original large RT-PCR fragments with sufficient redundancy that each nucleotide position
30 was confirmed by a minimum 3 independent M13 clones. The 5' and 3' terminal
genomic sequences of Ka and SF were converted to cDNA using the system for Rapid
Amplification of cDNA Ends (Life Technologies, Gaithersburg, MD) and sequenced by
automated sequencing.

These sequences are set forth in Figures 6A-6G (Ka) and Figures 7A-7G (SF), respectively. Analysis of these sequences revealed that the percent amino acid identity between HPIV3 and BPIV3 for each of the following proteins is: N (86%), P (65%), M (93%), F (83%), HN (77%), and L (91%). Thus sequence divergence was found distributed over many genes. The deduced amino acid sequence of the N genes of these two viruses is presented in GenBank #Af178654 (Ka) and #AF178655 (SF), not included. The position of the N ORF in the BPIV3 genome is indicated in the respective BenBank reports and included herein by reference. In the example below, the N ORF of the Ka or SF virus was initially selected for replacement of the corresponding gene in the HPIV3 virus because the N gene represents a gene with an intermediate level of sequence divergence among the six HPIV3 and BPIV3 proteins. In this study the N ORF, but not the 3' or 5' noncoding N gene sequences, was exchanged, which permitted us to assign an observed attenuation phenotype of cKa and cSF to the protein encoded by the N gene.

Human-bovine chimeric full-length PIV3 genomes were constructed by introducing the BPIV3 Ka or SF N coding region as a replacement for its HPIV3 counterpart into the rJS cDNA p3/7(131)2G which encodes a complete copy of HPIV3 positive-sense antigenomic RNA (see, e.g., Durbin et al., 1997a, *supra*; Hoffman et al., 1997, *supra*; Skiadopoulos et al., 1998, *supra*; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997; each incorporated herein by reference). BPIV3 and HPIV3 N coding regions with flanking sequences were first subcloned and further modified to permit an exchange of just the N ORF. pUC119JSN bearing the HPIV3 N gene and the plasmids with a BPIV3 N Ka or SF gene (pBSKaN and pBSSFN) were subjected to mutagenesis using the method of Kunkel (Proc. Natl. Acad. Sci. USA 82:488-492, 1985, incorporated herein by reference) to introduce NcoI and AflII restriction enzyme recognition sites at translational start and stop sites, respectively (Fig. 1A). Following NcoI/AflII digestion of pUC119KaN-NcoI/AflII, the BPIV3 N coding region was introduced as an NcoI/AflII fragment into pUC119JSN-NcoI/AflII as a replacement for the HPIV3 N coding region (Fig. 1 B). The chimeric N genes, which contain the HPIV3 3' and 5' noncoding sequences and the BPIV3 ORF, were modified by site-directed mutagenesis to restore the original HPIV3 noncoding sequence and BPIV3 coding sequence. This chimeric N gene was then introduced into the 5' half of the rJS antigenome, pLeft, in exchange for its corresponding

HPIV3 sequence (Figs. 2A and 2B) using existing MluI and EcoRI sites present in the human sequence. In each case parallel reactions were carried out for the SF N ORF. The chimeric pLeft plasmid was combined with the XhoI/NgoMI fragment from pRight containing the 3' half of the rJS antigenome flanked by the delta ribozyme and the T7 terminator at its 3' end (Fig. 2). The resulting chimeric PIV3 plasmids designated pB/HPIV3NKa or pB/HPIV3NSF, contained the full-length rJS antigenome in which the N ORF encoded the BPIV3 Ka or SF N protein.

Chimeric antigenomic HPIV3/BPIV3 cDNAs were transfected individually into HEp-2 cells grown to near-confluence in 6-well plates along with two previously-described support plasmids, pTM(P no C) and pTM(L), Lipofectace (Life Technologies, Gaithersburg, MD), and infected with a modified vaccinia virus recombinant that expresses bacteriophage T7 RNA polymerase (MVA-T7) as previously described (Durbin et al., *Virology* 234:74-83, 1997b). An N support plasmid used in previous work was omitted because the antigenomic plasmid expressed sufficient levels of the N protein. The cultures were maintained for 3.5 days at 32°C after which supernatants were harvested, passaged in LLC-MK2 cells and plaque-purified 3 times in LLC-MK2 cells. The identities of the chimeric viruses incorporating a human PIV 3 background genome or antigenome and a BPIV3 N protein (designated as rHPIV3-N_B chimeric recombinants or, more specifically, as "cKa" and "cSF" chimeric viruses) recovered from the transfections were confirmed by sequencing RT-PCR products containing the regions of the N ORF start and stop codons from virion RNA isolated after amplification of triply plaque-purified virus (Fig. 3). This amplified product and the corresponding amplified HPIV3 rJS and BPIV3 Ka or SF sequences were also subjected to TaqI digestion to confirm the chimeric identity of cKa and cSF viruses (Fig. 4). TaqI digestion profiles were distinct for the 3 parental and 2 chimeric viruses, and each parental profile included TaqI fragments of unique size, allowing the contribution of sequence of rJS, Ka and SF parents to the chimeric viruses to be verified. The recovered cKa and cSF chimeric recombinants each contained the expected sequences as designed.

EXAMPLE II

Replication of HPIV3/BPIV3 Chimeric Viruses in Cell Culture

Efficient replication of live attenuated virus vaccines in tissue culture cells is a feature of human-bovine chimeric PIV of the invention that permits efficient

manufacture of the recombinant vaccine materials. The multicycle replication of rJS parent, cKa, Ka parent, cSF, and SF parent in a bovine cell line (MDBK) and in a simian cell line (LLC-MK2) was determined by infecting cells with virus at a multiplicity of infection of 0.01 and harvesting samples (in triplicate) over a five day period of time (Fig. 5) as previously described (Tao et al., 1998, *supra*, incorporated herein by reference). The chimeric viruses replicated efficiently in both cell lines like their human or bovine parent viruses without significant delay in replication or a significant reduction in the titer of virus achieved. In each case, the chimeric viruses replicated to over $10^{7.0}$ TCID₅₀/ml which is well above the $10^{4.0}$ or $10^{5.0}$ dose of live attenuated human or bovine PIV vaccines currently being used in human clinical trials (Karron et al., 1996, *supra*; Karron et al., 1995a, *supra*; and Karron et al., 1995b, *supra*).

EXAMPLE III

Evaluation of Attenuation and Protective Efficacy of the HPIV3/BPIV3 Chimeric Viruses in Rhesus Monkeys

Both the SF and Ka BPIV3s are attenuated for the upper and the lower respiratory tract of the rhesus monkey (van Wyke Coelingh et al., 1988, *supra*). This attenuation phenotype correlates with attenuation in humans (Karron et al., 1995a, *supra*) as indicated by the fact that Ka is highly restricted in replication in the upper respiratory tract of fully susceptible seronegative infants and children. The absence of cough, croup, bronchiolitis, or pneumonia in the BPIV3-infected vaccinees suggests that the Ka BPIV3 virus is attenuated for the lower respiratory tract as well. Therefore, the rhesus monkey is widely accepted as a reasonably correlative model to evaluate attenuation of candidate PIV vaccine viruses and their efficacy against challenge with wild type PIV.

The rJS, cKa, Ka parent, cSF, and SF parent were administered intranasally and intratracheally at a dose of $10^{5.0}$ TCID₅₀ per site to rhesus monkeys. Replication was monitored using previously described procedures for obtaining samples from the upper (nasopharyngeal swab specimens) and lower (tracheal lavage specimens) respiratory tract and for titering the virus in LLC-MK2 cells (Hall et al., 1992, *supra*). The cKa and cSF recombinants were significantly attenuated for the upper respiratory tract (Table 1) exhibiting, respectively, a 63-fold or a 32-fold reduction in mean peak virus titer compared to that of the rJS HPIV3 parent. Both cKa and cSF were also attenuated for the lower respiratory tract, but this difference was only statistically significant for cSF. The low level of replication of rJS in the lower respiratory tract made

Table 1. Replication of cKa and cSFis restricted relative to HP1V3 in the upper and lower respiratory tracts of rhesus monkeys.

		Virus Replication					
Immu- nizing virus	No. of ani- mals	Mean titers				Mean peak titers	
		Log ₁₀ TCID ₅₀ /ml±standard error [Duncan grouping] ²				Log ₁₀ TCID ₅₀ /ml±standard error [Duncan grouping]	
		Nasopharynx		Trachea		Nasopharynx	Trachea
		day 6	day 7	day 4	day 6		
rJS	4	5.3±0.59[A]	3.9±0.36[A]	1.7±0.45[A]	1.7±0.29[A]	5.3±0.59[A]	2.5±0.51[A]
cKa	4	3.0±0.58[B]	2.9±0.42[AB]	1.5±0.40[A]	1.0±0.19[A]	3.5±0.54[B]	1.5±0.18[AB]
Ka	4	2.0±0.27[B]	2.4±0.30[B]	1.3±0.26[A]	1.3±0.21[A]	2.5±0.30[B]	1.6±0.15[AB]
cSF	4	3.3±0.40[B]	3.7±0.57[A]	1.1±0.25[A]	1.1±0.24[A]	3.8±0.46[B]	1.4±0.26[B]
SF	4	2.8±0.48[B]	2.6±0.40[AB]	1.6±0.46[A]	1.5±0.40[A]	3.3±0.28[B]	1.8±0.41[AB]

¹ Monkeys were inoculated intranasally and intratracheally with 10^{5.0} TCID₅₀ in 1 ml at each site.

² Mean viral titers in each column were assigned to statistically similar groups (designated with a letter) using a Duncan Multiple Range test ($\alpha = 0.05$). Mean titers in each column with different letters are statistically different.

The level of replication of each chimeric virus, cKa and cSF, was not significantly different from its bovine parent in the upper or the lower respiratory tract, although the chimeric viruses each replicated somewhat better than their BPIV3 parents in the upper respiratory tract. Thus, the acquisition of the N gene of either the Ka or SF BPIV3 by rJS HPIV3 attenuated the human virus for rhesus monkeys to a level approximately equivalent to that of the BPIV parent. Since the HPIV3/BPIV3 chimeric recombinants replicated efficiently in tissue culture cells *in vitro*, it is clear that the phenotype of host range restricted replication manifested by the two bovine parental viruses was transferred to HPIV3 by the N ORF. It is possible, but unknown and unpredictable, that substitution of other BPIV3 genes, such as M, P, or L, for their HPIV3 counterpart in rJS will give similar or greater levels of attenuation as observed upon substitution of the BPIV3 N gene for the HPIV3 N gene. The observation that the level of replication of cKa and cSF is slightly greater than that of their BPIV3 parents in the upper respiratory tract suggests that additional bovine genes contribute to the host range attenuation phenotype of the BPIV3 parent virus at this site.

Uninoculated monkeys and monkeys that were previously infected with a human or bovine PIV3 parental virus, or with the cKa or cSF chimeric virus, were challenged 42 days after the initial inoculation with $10^{6.0}$ TCID₅₀ of rJS intranasally and intratracheally in a 1 ml inoculum at each site. The nasopharynx and the trachea were sampled as described previously on the days indicated in Table 2. The titer of virus present at each site was determined for each monkey on LLC-MK2 cell monolayers, and the titers presented are mean peak titers (Hall et al., 1992, *supra*). Previous infection with either chimeric virus induced a high level of resistance to replication of the rJS challenge virus in both the upper and lower respiratory tract. Monkeys previously infected with cKa manifested a 300-fold reduction of replication of wild type HPIV3 (rJS) in the upper respiratory tract and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys. Monkeys previously infected with cSF manifested a 2000-fold reduction of replication of rJS in the upper respiratory tract and a 1000-fold reduction in the lower tract compared to uninoculated control monkeys. The level of reduction of replication of rJS challenge virus in monkeys previously-inoculated with cKa or cSF was comparable to that of monkeys previously infected with either the bovine or the human PIV parent. Thus, infection with either HPIV3/BPIV3 chimeric virus provided a high level of protection in the upper and lower respiratory tract of monkeys, and both chimeric viruses represent promising vaccine candidates.

Serum collected from monkeys on days 0 and 28 was tested by HAI assay using HPIV3 (JS strain) and BPIV3 (Ka strain) as antigen as previously described (Coelingh et al., *J. Infect. Dis.* 157:655-662, 1988). Although cKa-N and cSF-N were highly attenuated in the upper and lower respiratory tract of rhesus monkeys relative to rJS, each chimeric virus induced a hemagglutination-inhibiting (HAI) antibody response to HPIV3 that was 2.5 to 5-fold greater in magnitude than that induced by immunization with its respective BPIV3 patent. This likely is due to the presence of HPIV3 HN protein in the chimeric viruses. Furthermore, the HPIV3-specific HAI-responses induced by the chimeric viruses were statistically indistinguishable from that induced by immunization with rJS. An additional unexpected result demonstrated herein is that, following challenge of the monkeys with HPIV3, the level of HAI antibody in monkeys initially immunized with cKa-N or cSF-N was significantly greater than levels observed in animals immunized with rJS, Ka or SF.

Table 2. Rhesus monkeys previously infected with BPV3/HPV3 chimeric virus manifest a high level of resistance to replication of wt HPV3 challenge virus.

Initial infection (day 0) with ⁴	JS challenge virus titer in:			Serum HAI antibody titer ³ for HPIV3 on day of challenge (day 42) and 28 days later (day 70) ⁴	
	Nasopharyngeal swab ¹	Tracheal lavage ²			
	Number of animals	Mean peak titer ⁵ (Log ₁₀ TCID ₅₀ /ml±SE) [Duncan Grouping]	Mean peak titer ⁵ (Log ₁₀ TCID ₅₀ /ml±SE) [Duncan Grouping]		
none	4	5.1±0.22[A]	4.6±0.26[A]	≤1.0±0[D]	11.0±0[AB]
rJS	4	2.2±0.18[BC]	1.6±0.06[B]	9.2±0.25[AB]	9.5±0.29[C]
cKa-N	4	2.6±0.57[BC]	1.5±0[B]	9.8±0.25[A]	12.0±0[A]
Ka	4	3.0±0.4[B]	1.6±0.12[B]	7.5±0.5[C]	9.0±0.71[C]
cSF-N	4	1.8±0.16[C]	1.6±0.11[B]	9.8±0.25[A]	11.2±0.75[A]
SF	4	2.6±0.57[BC]	1.5±0[B]	8.5±0.29[B]	9.8±0.25[BC]

Note. Monkeys were challenged with JS intranasally and intratracheally (10⁶ TCID₅₀ in 1 ml at each site) 42 days after initial immunization.

¹ Nasopharyngeal swab samples were collected on days 3, 4, 5, 6, 7 and 8 post-challenge.

² Tracheal lavage samples were collected on days 4, 6 and 8 post-challenge.

³ HAI antibody titers are expressed as the mean reciprocal log₂±SE. Serum samples used for the HAI assay were collected on day of challenge (day 42) or on day 28 post-challenge (day 70).

⁴ All monkeys had a serum HAI titer to HPV3 of ≤1.0 prior to the initial infection on day 0.

⁵ The titer (TCID₅₀/ml) of virus present in each specimen was determined using LLC-MK2 cells, and the mean peak titers for four animals were determined as described in Table 1 and expressed as log₁₀TCID₅₀/ml±Standard Error (SE). Mean peak viral titers in each column were assigned to statistically similar groups (designated with a letter) using a Duncan Multiple Range test (α= 0.05). Mean titers in each column with different letters are statistically different.

EXAMPLE IV

Construction and Characterization of Chimeric HPIV3/BPIV3 Vaccine Candidates Having Heterologous Fusion And Hemagglutinin-Neuraminidase Glycoproteins

In the preceding example, the basis of host range restriction of replication of BPIV3 for the respiratory tract of primates was examined by the generation and characterization of a recombinant human PIV3 (rHPIV3) in which the N open reading frame (ORF) was replaced by that of its BPIV3 counterpart. The resulting chimeric virus, rHPIV3-N_B, also referred to as cKa or cSF, efficiently replicated in vitro but was restricted in replication in the upper respiratory tract of rhesus monkeys, identifying the N protein as an independent determinant of the host range restriction of BPIV3 in rhesus monkeys (Bailly et al., *J. Virol.* 74:3188-3195, 2000).

In the present example, the contribution of the fusion (F) and hemagglutinin-neuraminidase (HN) glycoprotein genes of bovine parainfluenza virus type 3 (BPIV3) to its restricted replication in the respiratory tract of non-human primates was examined by generating and characterizing two reciprocal chimeric BPIV3/HPIV3 viruses. A chimeric HPIV3 containing heterologous BPIV3 F and HN glycoprotein genes in place of its own, and the reciprocal recombinant comprising a BPIV3 "backbone" bearing the HPIV3 F and HN genes substituted for the counterpart BPIV3 glycoprotein genes, were generated to assess the effect of glycoprotein substitution on replication of HPIV3 and BPIV3 in the upper and lower respiratory tract of rhesus monkeys. Thus, in one chimeric virus, the F and HN genes of HPIV3 were replaced with their BPIV3 counterparts, resulting in a chimeric recombinant designated rHPIV3-F_BHN_B. The reciprocal chimeric recombinant PIV3 (rBPIV3-F_HHN_H) was constructed by replacing the F and HN genes of a recombinant BPIV3 (rBPIV3) with their HPIV3 counterparts. In the latter virus, the introduction of the HPIV3 F and HN ORFs into the BPIV3 backbone combines the antigenic determinants of HPIV3 with the backbone of BPIV3 and thus provides an improved vaccine candidate compared with parental BPIV3. The F and HN genes were exchanged as pairs in view of the proposed requirement for homologous HN and F proteins for parainfluenza viruses for full functional activity (Deng et al., *Virology* 209:457-469, 1995; and Tanabayashi et al., *J. Virol.* 70:6112-6118, 1996; each incorporated herein by reference).

The foregoing chimeric viruses were readily recovered and exhibited kinetics of replication in simian LLC-MK2 cells that were comparable to those of their parent viruses, suggesting that the heterologous glycoproteins were compatible with the

PIV3 internal proteins. The distinctive features of cytopathology of BPIV3 versus HPIV3 cosegregated with their respective F and HN genes. HPIV3 bearing the BPIV3 F and HN genes was attenuated for replication in rhesus monkeys to a level similar to that of its BPIV3 parent virus, indicating that the glycoprotein genes of BPIV3 are major
5 determinants of its host range restriction of replication in rhesus monkeys. BPIV3 bearing the HPIV3 F and HN genes (rBPIV3-F_HHN_H) replicated in rhesus monkeys to a level intermediate between that of HPIV3 and BPIV3.

These results indicate that the F and HN genes make a significant contribution to the overall attenuation of BPIV3. Furthermore, they demonstrate that
10 BPIV3 sequences outside the F and HN region also contribute to the attenuation phenotype in primates. This latter finding is consistent with the demonstration in the preceding example that the nucleoprotein coding sequence of BPIV3 is a determinant of its attenuation for primates. Despite its restricted replication in the respiratory tract of rhesus monkeys, rBPIV3-F_HHN_H conferred a level of protection against challenge with
15 wild type HPIV3 that was indistinguishable from that conferred by previous infection with wild type HPIV3. From these and related findings, the usefulness of rBPIV3-F_HHN_H as a vaccine candidate against HPIV3 is readily apparent.

Viruses and cells

20 HEp-2 and simian LLC-MK2 monolayer cell cultures were maintained in MEM medium (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 50ug/ml gentamicin sulfate, and 4mM glutamine (Life Technologies, Gaithersburg, MD).

The wild type BPIV3 strain Kansas/15626/84 (Clone 5-2-4, Lot BPI3-1)
25 (BPIV3 Ka), the HPIV3 JS wild type, its recombinant version (rHPIV3), and the rHPIV3 virus containing the BPIV3 Ka N ORF in place of the HPIV3-N ORF (rHPIV3-N_B) are each described above (see also, Clements et al., 1991, *supra*; Karron et al., 1995a, *supra*; Bailly et al., 2000, *supra*; and Durbin et al., 1997, *supra*). PIVs were propagated at 32°C in LLC-MK2 cells (ATCC CCL-7), as previously described (Hall et al., 1992, *supra*).

30 The modified vaccinia strain Ankara (MVA) recombinant virus that expresses bacteriophage T7 RNA polymerase is described by Wyatt et al. (1995, *supra*).

Construction of antigenomic cDNAs encoding recombinant BPIV3/HPIV3 viruses.

a) Construction of cDNA to recover rBPIV3

A full length cDNA was constructed to encode the complete 15456 nucleotide (nt) antigenomic RNA of BPIV3 Ka, as described above. The cDNA was assembled from 4 subclones derived from reverse transcription (RT) of viral RNA using the SuperScript II Pre-amplification System (Life Technologies, Gaithersburg, MD) and polymerase chain reaction (PCR) amplification with a High Fidelity PCR kit (Clontech Laboratories, Palo Alto, CA). The RT-PCR products were cloned into modified pUC19 plasmids (New England Biolabs, Beverly, MA) using the following naturally occurring internal restriction enzyme recognition sites: *Sma* I (BPIV3 Ka sequence position nt186), *Pst* I (nt 2896), *Mlu* I (nt 6192), *Sac* II (nt 10452) and *Bsp* LU11 (nt 15412). Multiple subclones of the antigenomic cDNA were sequenced using a Perkin Elmer ABI 310 sequencer with dRhodamine Terminator Cycle Sequencing (Perkin Elmer Applied Biosystems, Warrington, UK), and only those matching the consensus sequence of BPIV3 Ka were used for assembly of the full length clone. The 3' and 5' ends of BPIV3 Ka were cloned and the assembly of the full length cDNA took place in the previously described p(Right) vector (Durbin et al., 1997, *supra*), which we modified to contain a new polylinker with restriction enzyme recognition sites for *Xho* I, *Sma* I, *Mlu* I, *Sac* II, *Eco* RI, *Hind* III and *Rsr*II. The full length cDNA clone pBPIV3(184) contained the following elements in 3' to 5' order: a T7 promoter followed by 2 non-viral guanosine residues, the complete antigenomic sequence of BPIV3 Ka, a hepatitis delta virus ribozyme and a T7 polymerase transcription terminator (Bailly et al., 2000, *supra*; and Durbin et al., 1997a, *supra*).

b) Construction of rHPIV3-F_BHN_B and rBPIV3-F_HHN_H

Unique restriction enzyme recognition sites were introduced into the BPIV3 antigenomic cDNA and into the previously described HPIV3 antigenomic cDNA p3/7(131)2G (Durbin et al., 1997a, *supra*) to facilitate the exchange of the F and HN genes between BPIV3 and HPIV3 cDNAs. Using the transformer site-directed mutagenesis protocol from Clontech (Clontech Laboratories, Palo Alto, CA), *Sgr*AI restriction sites were introduced in the downstream non-coding region of the M gene at position 4811 of the rBPIV3 sequence and position 4835 of the rHPIV3 JS sequence (GenBank accession # Z11575). The nucleotide number given for the position of restriction enzyme recognition sites indicates the nucleotide after which the enzyme cuts, not the first nucleotide of the restriction enzyme recognition site. The sequence was

changed from TCCAACATTGCA (SEQ. ID. NO. 2) to TCCACCGGTGCA (SEQ. ID. NO. 3) in rBPIV3 and from CGGACGTATCTA (SEQ. ID. NO. 4) to CGCACCGGTGTA (SEQ. ID. NO. 5) in rHPIV3 (recognition sites underlined). *Bsi*WI restriction sites were introduced in the downstream non-coding region of the HN gene at nt 8595 of the rBPIV3 sequence and at nt 8601 of the rHPIV3 JS sequence. The sequence was changed from GATATAAAGA (SEQ. ID. NO. 6) to GACGTACGGA (SEQ. ID. NO. 7) in rBPIV3 to give pBPIVs(107) and from GACAAAAGGG (SEQ. ID. NO. 8) to GACGTACGGG (SEQ. ID. NO. 9) in rHPIV3 to give pHPIVs(106). The F and HN genes were exchanged between pBPIVs(107) and pHPIVs(106) by digestion of each with *Sgr*AI and *Bsi*WI, gel purification of the fragments, and assembly of the appropriate fragments into the two full length cDNAs. The HPIV3 backbone bearing the BPIV3 F and HN genes, designated pHPIV(215), encoded 15480 nts of viral sequence, of which nts 4835 to 8619 came from BPIV3, and it was used to derive rHPIV3-F_BHN_B (Figures 8A-8C). The BPIV3 backbone bearing the HPIV3 F and HN genes, designated pBPIV(215), encoded 15438 nts of viral sequence, of which nts 4811 to 8577 came from HPIV3, and it was used to derive rBPIV3-F_HHN_H (Figured 8A-8C).

BPIV3 support plasmids for recovery of virus from cDNA.

Support plasmids encoding the BPIV3 Ka N, P and L genes were assembled in modified pUC19 vectors and then cloned into the previously described pTM-1 vector (Durbin et al., 1997a, *supra*). In order to place the individual genes immediately downstream of the T7 promoter in the pTM vector, an *Nco* I site was introduced at the start codon of the N, P and L open reading frames (ORFs) using site-directed mutagenesis. The *Nco* I restriction site and a naturally occurring restriction site downstream of each ORF (*Spe* I for N, *Hinc*II for P and *Bsp* LU11I for L) was used for cloning into pTM. After cloning, the *Nco* I site in pTM(N) was mutagenized back to the original sequence to restore the correct amino acid assignment in the second codon. In pTM(P) and pTM(L) the amino acid sequence encoded by the ORF was not altered by the introduction of *Nco* I sites.

Transfection.

HEp-2 cells (approximately 1.5×10^6 cells per well of a six-well plate) were grown to 90% confluence and transfected with 0.2 μ g each of the BPIV3 support plasmids pTM(N) and pTM(P), and 0.1 μ g of pTM(L), along with 5 μ g of the full length

antigenomic cDNA and 12µl LipofectACE (Life Technologies, Gaithersburg, MD). Each transfection mixture also contained 1.5×10^7 plaque forming units (PFU) of MVA-T7, as previously described (Durbin et al., 1997, *supra*). The cultures were incubated at 32°C for 12 hrs before the medium was replaced with MEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum. The supernatants were harvested after incubation at 32°C for an additional three days, and were passaged onto LLC-MK2 cell monolayers in 25cm² flasks and incubated for 5 days at 32°C. Virus present in the supernatant was plaque-purified three times prior to amplification and characterization.

10 **Molecular characterization of recovered chimeric recombinants.**

The presence of the heterologous F and HN genes in the bovine or human PIV3 backbone was confirmed in plaque-purified recombinant viruses by RT-PCR of viral RNA isolated from infected cells or supernatant, which was performed using a primer pair that recognizes conserved sequences in rBPIV3 and rHPIV3. This yielded similarly sized fragments (nts 4206-9035 in rBPIV3, nts 4224-9041 in rHPIV3, nts 4206-9017 in rBPIV3-F_HHN_H, and nts 4224-9059 in rHPIV3-F_BHN_B) which were then digested with *Eco* RI and analyzed by electrophoresis on a 1% agarose gel (Fig. 9). The nucleotide sequence flanking the introduced *Sgr*AI and *Bsi*WI restriction sites in each virus was confirmed by sequencing the respective RT-PCR product.

20 **Replication of HPIV3/BPIV3 chimeric viruses in cell culture.**

The multicycle growth kinetics of BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, rHPIV3-N_B and rHPIV3 in LLC-MK2 cells were determined by infecting cells in triplicate at a multiplicity of infection (MOI) of 0.01 and harvesting samples at 24 hr intervals over a six day period, as previously described (Tao et al., 1998, *supra*). Samples were flash-frozen and titered in a single assay on LLC-MK2 cell monolayers in 96 well plates at 32°C, as described (Durbin et al., *Virology* 261:319-330, 1999b, incorporated herein by reference).

30 **Primate Model Studies.**

Rhesus monkeys seronegative for PIV3 as determined by hemagglutination-inhibition (HAI) assay (van Wyke Coelingh et al., 1988, *supra*) were inoculated intranasally and intratracheally in groups of 2 or 4 animals with 10^5 tissue

culture infectious dose₅₀ (TCID₅₀) per ml of BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, rHPIV3-N_B or rHPIV3. Nasopharyngeal swabs were collected daily on days 1 to 11 and on day 13. Tracheal lavage samples were collected on days 2, 4, 6, 8, and 10 post-infection. Individual samples were flash-frozen and stored at -70°C until all samples were available for titration. Virus in the specimens was titrated on LLC-MK2 cell monolayers in 24 and 96 well plates as previously described (Durbin et al., 1999b, *supra*). Sera collected from monkeys on days 0 and 28 was tested by HAI assay using HPIV3 JS and BPIV3 Ka as antigens, as previously described (van Wyke Coelingh et al., 1988, *supra*). On day 28 post inoculation, the monkeys were challenged intranasally and intratracheally with 10⁶ TCID₅₀ per site of HPIV3 JS. Nasopharyngeal swab samples were collected on days 3, 4, 5, 6, 7 and 8, and tracheal lavage samples on days 4, 6 and 8 post challenge. Samples were titrated in a single assay as described above. Serum was collected on day 28 post challenge.

15 **Recovery of rBPIV3 and BPIV3/HPIV3 chimeric viruses (rHPIV3-F_BHN_B and rBPIV3-F_HHN_H) from cDNA.**

A complete BPIV3 antigenomic cDNA, designated pBPIV(184), was constructed to encode the consensus sequence of BPIV3 Ka. This BPIV3 antigenomic cDNA was further modified by the introduction of unique *SgrAI* and *BsiWI* sites into the downstream noncoding region of the M and HN genes, respectively (Fig. 8C). The same restriction sites were introduced into the downstream noncoding region of the M and HN genes of a previously described complete HPIV3 antigenomic cDNA, p3/7(131)2G (Durbin et al., 1997a, *supra*). The F and HN glycoprotein genes of HPIV3 and BPIV3 were swapped by exchanging this *SgrAI*-*BsiWI* restriction fragment. A direct exchange of entire genes was anticipated to be well-tolerated because of the high level of sequence conservation between the cis-acting signals of BPIV3 and HPIV3. The HPIV3 antigenomic cDNA bearing the BPIV3 F and HN genes was designated pHPIV(215), and the BPIV3 antigenomic cDNA bearing the HPIV3 F and HN genes was designated pBPIV(215).

30 The antigenomic cDNAs pBPIV(184), pHPIV(215), pBPIV(215) and p3/7(131)2G were separately transfected into HEp-2 cells along with the three BPIV3 support plasmids pTM(N), pTM(P) and pTM(L), and the cells were simultaneously infected with recombinant MVA expressing the T7 RNA polymerase. To confirm that

the recovered viruses indeed were the expected rBPIV3, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H and rHPIV3 viruses, intracellular RNA or RNA from supernatant from each cloned virus was analyzed by RT-PCR using a primer pair that recognized identical sequences in HPIV3 JS and BPIV3 Ka. The primer pair amplified a 4.8 kb fragment of DNA

5 corresponding to the downstream end of the M gene, the F and HN genes, and the upstream end of the L gene (nts 4206-9035 in rBPIV3, nts 4224-9041 in rHPIV3, nts 4206-9017 in rBPIV3-F_HHN_H, and nts 4224-9059 in rHPIV3-F_BHN_B). The generation of each PCR product was dependent upon the inclusion of reverse transcriptase, indicating that each was derived from viral RNA and not from contaminating cDNA (data not

10 shown). The PCR products were then digested with *Eco* R1, which would be predicted to yield a different, unique restriction enzyme digest pattern for each of the four viruses (Figure 9). In each case, the predicted pattern was observed, confirming the identity of the backbone and the inserted F and HN genes. In addition, nucleotide sequencing was performed on the RT-PCR products to confirm the presence of the introduced restriction

15 sites and flanking sequences.

The cytopathic effect (CPE) caused by rBPIV3-F_HHN_H in LLC-MK2 cells was indistinguishable from that of HPIV3 JS (condensed, rounded-up cells and small syncytia) but different from BPIV3 (large multicellular syncytia), whereas the CPE caused by rHPIV3-F_BHN_B was identical to that caused by the BPIV3. This indicates that

20 the cytopathology of the chimeric PIVs cosegregated with the parental origin of the F and HN genes.

BPIV3/HPIV3 chimeric viruses replicate efficiently in cell culture.

The growth kinetics of rHPIV3-F_BHN_B and rBPIV3-F_HHN_H were

25 compared with that of their parental viruses by infecting LLC-MK2 monolayers at an MOI of 0.01 and monitoring the production of infectious virus. The kinetics and magnitude of replication of the two chimeric viruses were comparable to those of their HPIV3 or BPIV3 parental viruses (Figure 10). This suggested that BPIV3 and HPIV3 glycoproteins were compatible with the heterologous PIV3 internal proteins. This is an

30 important property because it will be possible to efficiently prepare vaccine virus.

The F and HN genes of the BPIV3/HPIV3 chimeric viruses are determinants of the host range restriction of replication of BPIV3 Ka in the respiratory tract of rhesus monkeys.

rHPIV3-F_BHN_B and rBPIV3-F_HHN_H were evaluated for their ability to replicate in the upper and lower respiratory tract of rhesus monkeys. In particular, the effects of introduction of the BPIV3 F and HN genes into HPIV3 on attenuation of replication in rhesus monkeys was demonstrated, as described above for the BPIV3 N protein (see also, Bailly et al., 2000, *supra*). In addition, the effects of introduction of the HPIV3 F and HN genes into BPIV3 on replication in rhesus monkeys was determined. If the predominant attenuating mutations of BPIV3 were in genes other than the F and HN, then one would expect little overall effect of the HPIV3-BPIV3 glycoprotein exchange on replication of BPIV3 in rhesus monkeys.

Each chimeric virus was administered intranasally and intratracheally to rhesus monkeys at a dose of 10⁵ TCID₅₀ per site. The level of replication of the chimeric viruses was compared to that of the rHPIV3 and BPIV3 parental viruses and to that of rHPIV3-N_B (Table 3). Since the rHPIV3 parental virus replicated to a low to moderate level in the lower respiratory tract, meaningful comparisons between groups could only be made for replication in the upper respiratory tract. The level of replication of rHPIV3-F_BHN_B was similar to that of its BPIV3 parent and substantially lower than that of its HPIV3 parent (Table 3; Figure 11, panel A). This showed that the BPIV3 glycoprotein genes contained one or more major determinants of the host range attenuation phenotype of BPIV3 for rhesus monkeys. The magnitude and pattern of replication of rHPIV3-F_BHN_B and rHPIV3-N_B were very similar, indicating that each of the two bovine genetic elements, namely the N gene versus the F and HN genes, attenuate HPIV3 to a similar extent.

Table 3. The F and HN glycoprotein genes of BPIV3 contribute to its restricted replication in the respiratory tract of rhesus monkeys.

Immunizing virus ¹	Number of animals ²	Mean peak virus titer ³		Serum HAI antibody titer (mean recip. log ₂ ± S.E.) for HPIV3 on day 28 ⁷	Serum HAI antibody titer (mean recip. log ₂ ± S.E.) for BPIV3 on day 28 ⁷
		NP swab ⁵	Tracheal lavage ⁶		
rHPIV3	6	4.7±0.54 [A]	2.4±0.37 [A]	9.5±0.72 [A]	6.8±1.03 [B]
rBPIV3-F _H HN _H	4	3.1±0.58 [B]	1.6±0.05 [A]	6.8±0.63 [BC]	3.8±0.63 [C]
rHPIV3-N _B	6	3.0±0.60 [B]	1.4±0.19 [A]	8.2±0.48 [AB]	6.5±0.62 [B]
rHPIV3-F _B HN _B	4	2.9±0.28 [B]	2.0±0.24 [A]	4.5±0.29 [D]	9.5±0.65 [A]
BPIV3 Ka	6	2.6±0.26 [B]	1.6±0.10 [A]	5.5±0.62 [CD]	9.2±0.60 [A]

¹ Monkeys were inoculated intranasally and intratracheally with 10⁵ TCID₅₀ of virus in a 1 ml inoculum at each site.

² The groups with 6 animals contain 4 animals each from a previous rhesus study (Bailey et al., 2000, *supra*).

³ Mean of the peak virus titers for each animal in its group irrespective of sampling day. S.E. = standard error.

⁴ Virus titrations were performed on LLC-MK2 cells at 32°C. The limit of detectability of virus titer was 10 TCID₅₀/ml. Mean viral titers were compared using a Duncan Multiple Range test ($\alpha=0.05$). Within each column, mean titers with different letters are statistically different. Titrers indicated with two letters are not significantly different from those indicated with either letter.

⁵ Nasopharyngeal swab samples were collected on days 1 to 11 and on day 13.

⁶ Tracheal lavage samples were collected on days 2, 4, 6, 8 and 10 post-infection.

⁷ The titers on day 0 were <2.0. Day 28 was the day of challenge with wild type HPIV3.

**Two of the animals in the rHPIV3 group were infected with rHPIV3s, the virus containing two restriction enzyme recognition sites for the glycoprotein swap.

The rBPIV3-F_HHN_H chimeric virus replicated significantly less well than rHPIV3 (Table 3), and it grouped with BPIV3 in a Duncan multiple range test. However, inspection of its pattern of replication in Figure 11B suggested that rBPIV3-F_HHN_H replicated to a level intermediate between that of its HPIV3 and BPIV3 parents. The interpretation that rBPIV3-F_HHN_H replicates to a level intermediate between that of its parents is supported by Friedman's test of consistency of ranks (Sprent, P., "A Generalization Of The Sign Test," Applied Nonparametric Statistical Methods, pp. 123-126, Chapman and Hall, London, 1989, incorporated herein by reference), which indicated that the median titers of HPIV3, rBPIV3-F_HHN_H, and BPIV3 between day 3 and day 8 post infection are significantly different (d.f.2,8; p<0.05). The observation that the introduction of the HPIV3 F and HN proteins resulted in an increase in the replication of BPIV3 in rhesus monkeys indicates (i) that F and HN contain one or more determinants of host range restriction and (ii) that one or more genetic elements of BPIV3 that lie outside of the F and HN genes, e.g. the N protein, attenuate the virus for rhesus monkeys. This confirms that the genetic basis for host range restriction can involve multiple genes.

The chimeric BPIV3 bearing HPIV3 glycoprotein genes induces serum HAI antibody to HPIV3 and a high level of resistance to wt HPIV3 challenge.

rBPIV3-F_HHN_H has important features that make it a candidate live attenuated virus vaccine against HPIV3, including attenuating genes from BPIV3 and the antigenic specificity of HPIV3, i.e. the F and HN glycoproteins, which are the major protective antigens. Therefore, its immunogenicity and protective efficacy against challenge with HPIV3 were documented. Rhesus monkeys were immunized by infection with BPIV3 Ka, rHPIV3-F_BHN_B, rBPIV3-F_HHN_H, rHPIV3-N_B, or rHPIV3. They were challenged 28 days later with HPIV3 JS wild type virus. Serum samples were taken prior to the initial infection on day 0 and prior to the challenge. BPIV3 and rHPIV3-F_BHN_B induced serum HAI antibodies that reacted more efficiently with BPIV3 than HPIV3, whereas the converse was the case for HPIV3 and rBPIV3-F_HHN_H. Thus, the origin of the glycoprotein genes in each virus determined whether the HAI antibody response was directed predominantly against HPIV3 or against BPIV3. The replication of challenge HPIV3 virus was significantly reduced in the upper and lower respiratory tract of previously immunized monkeys (Table 4). Although the level of protective efficacy against HPIV3 was not significantly different among the different viruses,

viruses bearing HPIV3 F and HN were consistently more protective in the upper respiratory tract than were viruses bearing BPIV3 F and HN. This is in accordance with the higher level of HPIV3-specific serum HAI antibodies induced by viruses bearing HPIV3 F and HN.

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Table 4. Immunization of rhesus monkeys with BPIV3/HPIV3 chimeric recombinant virus induces resistance to challenge with wild type HPIV3

Immunizing virus ¹	Number of animals ²	Mean peak virus titer ³		Serum HAI antibody	
		(log ₁₀ TCID ₅₀ /ml ± S.E.) [Duncan Grouping] ⁴	Tracheal lavage ⁶	titer (mean recip. log ₂ ± S.E.) for HPIV3 on the day of challenge	Serum HAI antibody titer (mean recip. log ₂ ± S.E.) for HPIV3 28 days after challenge
none	4	4.5±0.33 [A]	4.5±0.19 [A]	<2	12.0±0.58 [A]
rHPIV3	6	2.3±0.14 [B]	1.2±0.20 [B]	9.5±0.72 [A]	11.7±0.21 [A]
rBPIV3-F _H HN _H	4	2.5±0.25 [B]	1.0±0.48 [B]	6.8±0.63 [BC]	10.5±0.29 [AB]
rHPIV3-N _B	6	2.3±0.41 [B]	1.4±0.08 [B]	8.2±0.48 [AB]	11.5±0.22 [A]
rHPIV3-F _B HN _B	4	3.0±0.14 [B]	1.0±0.0 [B]	4.5±0.29 [D]	9.5±0.87 [B]
BPIV3 Ka	6	2.9±0.26 [B]	1.3±0.20 [B]	5.5±0.62 [CD]	9.3±0.76 [B]

¹ Each previously immunized monkey and non-immunized controls were challenged with 10⁶ TCID₅₀ of HPIV3 JS in a 1 ml inoculum at each site 28 days after immunization.

² The groups with 6 animals contain 4 animals each from a previous rhesus study (Bailly et al., 2000, *supra*).

³ Mean of the peak virus titers for each animal in its group irrespective of sampling day.

⁴ Virus titrations were performed on LLC-MK2 cells. The limit of detectability of virus titer was 10 TCID₅₀/ml. Mean viral titers were compared using a Duncan Multiple Range test ($\alpha=0.05$). Within each column, mean titers with different letters are statistically different. Titers indicated with two letters are not significantly different from those indicated with either letter. The group of unimmunized animals were not included in the Duncan analysis at the day of challenge.

⁵ Nasopharyngeal swab samples were collected on days 3 to 8 post challenge.

⁶ Tracheal lavage samples were collected on days 4, 6 and 8 post challenge.

**Two animals in the rHPIV3 group were infected with rHPIV3s.

Based on the foregoing examples, the invention provides for importation of BPIV genes into a virulent HPIV backbone and visa versa to yield novel, human-bovine chimeric PIV vaccine candidates. In exemplary chimeric recombinants disclosed in the present example, rBPIV3-F_HHN_H and its rHPIV3-F_BHN_B counterpart, replicated in vitro as well as the respective parental viruses. It was also confirmed that the F and HN exchange between the BPIV3 and HPIV3 is compatible since the considerably more divergent HPIV1 F and HN proteins were highly functional in a HPIV3 background (Tao et al., J. Virol. 72:2955-2961, 1998), which was evinced by the undiminished capacity of the chimeric viruses for replication in vitro. rBPIV3-F_HHN_H replicated in the upper respiratory tract of rhesus monkeys to a level intermediate between that of its HPIV3 and BPIV3 parents indicating that the BPIV3 F and HN genes make an independent contribution to the overall attenuation of BPIV3 for primates. The overall attenuation of BPIV3 virus thus is the sum of two or more genetic elements, one of which is the set of F and HN genes and one of the others is indicated to be N.

Although BPIV3 itself is being evaluated as a vaccine virus for HPIV3 (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; and Karron et al., J. Infect. Dis. 171:1107-1114, 1995), it is only 25% related antigenically to HPIV3 (Coelingh et al., J. Infect. Dis. 157:655-662, 1988). Thus, the immunogenicity of BPIV3 against HPIV3 will be improved if it is modified according to the present invention to express the protective F and HN antigens of HPIV3. rBPIV3-F_HHN_H represents such a virus, and, in the present example, immunization of rhesus monkeys with rBPIV3-F_HHN_H induced a higher level of antibody to HPIV3 than did immunization with BPIV3. Furthermore, rBPIV3-F_HHN_H conferred a level of protection against replication of HPIV3 challenge in the upper and lower respiratory tract that was statistically indistinguishable from that conferred by a previous infection with rHPIV3. Similarly, rHPIV3-N_B, which is attenuated by the BPIV3 N protein but possesses HPIV3 protective antigens, also induced a high level of resistance to HPIV3 challenge. Despite replicating to similar levels in rhesus monkeys, rHPIV3-N_B induced higher levels of antibodies to HPIV3 than rBPIV3-F_HHN_H.

rBPIV3-F_HHN_H replicates to higher levels in rhesus monkeys than BPIV3, although it is significantly attenuated compared to HPIV3. Since the level of replication of BPIV3 in humans is low (Karron et al., J. Infect. Dis. 171:1107-1114, 1995), this increase is expected to be well tolerated among vaccinees. Alternatively, additional methods to attenuate human-bovine chimeric viruses of the invention are disclosed herein

to ensure that the vaccine viruses replicate only to moderate levels, for example in human infants, to prevent unacceptable respiratory tract illness among vaccinees. Within other aspects of the invention, the slight increase in replication of rBPIV3-F_HHN_H in primates offers an opportunity to use rBPIV3-F_HHN_H as a vector for heterologous viral antigens
5 such as glycoproteins of other PIVs (e.g., HPIV1 and HPIV2), the RSV F and G glycoproteins, and the measles HA glycoprotein, which can be incorporated as added or substituted gene(s) or genome segment(s) into the attenuated HPIV3 vaccine candidate. In various alternative embodiments disclosed herein, the slight increase in replication of rBPIV3-F_HHN_H in monkeys over that of BPIV3 can be offset by the addition of foreign
10 viral protective antigens, e.g., RSV glycoproteins, whose addition provides a selected level of attenuation. The data presented here further defined the basis for the host range restriction of BPIV3 for primates and identify rBPIV3-F_HHN_H as a potential vaccine candidate against HPIV3 and as a vector for heterologous viral antigens.

15 Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications are comprehended by the disclosure and may be practiced without undue experimentation within the scope of the appended claims, which are presented by way of illustration not limitation.